

**Exploring Natural Product Biosynthesis in *Photorhabdus laumondii*: A Novel Strategy through Activation of Bacterial Enhancer Binding Proteins**

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## Abstract

# Exploring Natural Product Biosynthesis in *Photorhabdus laumondii*: A Novel Strategy through Activation of Bacterial Enhancer Binding Proteins

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It has been reported that the number of known natural products is much less than the number of biosynthetic gene clusters (BGCs) found in the genomes of microorganisms, which suggests a considerable potential for new drug discovery. However, a significant challenge arises as these BGCs cannot be induced in a laboratory setting. Due to high fitness cost for bacteria, these encoded natural products are tightly regulated. Consequently, our limited understanding of the triggers and regulatory mechanisms of these BGCs impedes progress in the search for potential antibiotics.

To tackle this issue, we propose an alternative approach distinct from the traditional methods such as high-throughput screening, heterologous expression, or the introduction of constitutive promoters. Our strategy involves using the bacterial machinery to activate the expression of BGCs by constitutively activating their bacterial enhancer binding proteins (bEBPs). This is achieved by deleting the regulatory domain located at the N-terminal of bEBPs. The majority of these bEBPs are responsible for activating the  $\sigma^{54}$ -RNA polymerase holoenzyme, thereby initiating transcription.

We have demonstrated  $\sigma^{54}$ 's importance in regulating the expression of a wide range of natural products in *Photorhabdus laumondii*. This was achieved by utilizing the core domain of DctD (DctD<sub>(141-394)</sub>), a bEBP from *Sinorhizobium meliloti*. Further examination of the modified *P. laumondi* bEBP (mEBPs) revealed diverse effects on natural product expression involving activation and/or repression. Some of these mEBPs exhibit elevated expression levels and, in certain instances, demonstrate propensity for specific natural products compared to DctD<sub>(141-394)</sub>. These preliminary findings suggest that mEBPs, could in time enable us to selectively express a subset of  $\sigma^{54}$ -dependent BGCs. This would bypass the need for specific growth conditions, unlocking a reservoir of previously inaccessible natural products.

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## **Contributions of Authors**

Lydia Rili: Wrote the first draft of this thesis and corrected subsequent versions. Performed all listed experiments and analyses.

Brandon Findlay: Project supervisor responsible for correcting the manuscript. Performed an antiSMASH analysis that identified the bacterial strain used in this study.

## **Copyright information**

The data presented in this thesis will be adapted for a publication in a near future.

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## **List of abbreviation**

- NP Natural Product
- BGC Biosynthetic Gene Cluster
- bEBP bacterial Enhancer Binding Protein
- dsDNA double-stranded DNA
- DctD C<sub>4</sub>-dicarboxylic acid transport protein D
- mEBP modified EBP
- R domain Regulatory domain
- C domain Core (AAA+) domain
- D domain DNA binding domain
- LB/LA Lysogeny Broth/Agar
- TSB/TSA Tryptic Soy Broth/Agar
- PCR Polymerase Chain Reaction
- cPCR colony PCR
- ON Overnight
- WT Wild Type
- LC-MS Liquide Chromatography Mass Spectrometry
- MVAP-A Mevalagmapeptide A
- PL-A Phurealipid A
- PPY-D Photopyrone D
- IPS Isopropylstilbene
- AQ 270 Anthraquinone 270
- GXP-A GameXpeptide A
- RT Retention Time
- PH Peak Hight
- Y Yellow
- B Beige

# INTRODUCTION

## Natural products

Natural products (NPs) which include secondary metabolites, are small molecules or proteins that are considered non-essential for growth but crucial for development under stressful conditions<sup>1,2</sup>. In response to various environmental challenges, microorganisms have adapted to produce a diverse range of NPs. These challenges include the need for communication, both intra- or interspecies to regulate a colony-wide response to the surrounding stimuli<sup>3</sup>. An illustration of that is evident in the symbiotic interaction of bacteria with its host, which may lead to fostering host growth<sup>4,5</sup>. Nonetheless, our interest in these NPs lies in their bioactive properties, such as their ability to inhibit, deter or kill similar or closely related microbes perceived as competitors or prey<sup>1,2,4,6,7</sup>.

## NPs, Advantages and Drawbacks

In pharmacology these bioactive NPs are very important. They are used in modern medicine as antibiotics, biocontrol agents against food spoilage by fungi, as well as therapeutic treatments like anti-cancer drugs and immunosuppressants<sup>2,4,8-10</sup>.

Our primary objective behind the development of different antibiotics is to combat bacterial infections<sup>11</sup>. However, as our usage of antibiotics increases, so does the surge in multidrug bacterial resistance<sup>8,12</sup>. We can observe this resistance through the different bacterial strategies implemented in response to this new hostile environment. These strategies include horizontal acquisition of resistance genes and local modification of foreign molecules<sup>5,11</sup>. However, the most common mechanism of resistance is up-regulation of specific genes encoding

for transporters or permeases as well as efflux pumps to remove antibiotics that permeated the bacterial cell wall<sup>5,11,13</sup>. Combined, these approaches limit the transport of antibiotics into the cell, providing broad-spectrum resistance at minimal cost.

It is predicted that by 2050 the leading cause of death would be through bacterial infections which would be permitted to proliferate due to antibiotic resistance<sup>12</sup>. The life losses would be numbered in the tens of millions, with a considerable impact on our health care system and livelihood<sup>12</sup>. Hence, the renewed global interest in further exploring the bacterial genome and identifying new NPs with antibacterial activity<sup>8,9</sup>.

### **NPs and their Biosynthetic Gene Clusters**

In most complex eukaryotes, each gene falls under the control of complex levels of regulation to obtain one final protein product<sup>14</sup>. Prokaryotes, on the other hand express their genes as a group under the control of one set of regulatory elements to yield multiple proteins<sup>14</sup>. These secondary metabolite-encoding genes are physically clustered by function in packages referred to as biosynthetic gene clusters (BGCs). These functions include regulation, co-factor biosynthesis, transport elements and self-resistance, all expressed for the production and the proper function of multiple bioactive NPs and/or proteins at once.<sup>14-16</sup>

Some of these BGCs are believed to be cryptic. Their cognate NPs have yet to be identified since they are difficult to be expressed in standard laboratory growth conditions<sup>8</sup>. This issue is caused by either heavy regulation, expression below our detection limit, or more importantly we have yet to uncover all the cues and triggers required for their activation.<sup>1,8,9,17,18</sup>

## **NPs potential: Exploring Conventional Methods**

Many traditional technologies focus on different aspect of this issue. To mine for new NPs, some allow for a successful reading of the BGCs and others for the identification of some components through large screening<sup>19</sup>. Amongst these technologies are:

High-throughput screening. In this approach, libraries of millions of compounds are tested with the aim of identifying elicitors that would successfully induce silent promoters<sup>19,20</sup>. According to Mao et al. this approach is typically carried out by introducing a reporter gene downstream of a silent native promoter and exposing the modified strain to multiple elicitors independently. The goal as stated before is to identify one or more molecules that can be used as an inducer on the native strain to trigger the targeted BGC.<sup>20</sup>

Heterologous expression. This strategy consists of constructing a vector carrying a cryptic BGC (otherwise silent in its native strain) and transforming it into a heterologous host, which was genetically modified usually through genome minimization or metabolite simplification (usually *Escherichia coli*) to elicit its expression. Upon obtaining the cognate NP(s), bioassay testing is conducted to determine the toxicity of the compound(s).<sup>4,21–23</sup>

Promotor swapping. This approach involves the introduction of a constitutive promoter upstream of the cryptic BGC and controllably inducing its expression. This technique requires that the genome of the explored strain be already annotated and sequenced.<sup>20</sup>

These strategies have helped to unlock the NP potential of many strains. However, they are time-consuming, and most of them overlook key molecules found in the native strains. These molecules could be substrates or co-factors that are required for the proper activation of these cryptic BGCs and functioning of their proteins.<sup>1,17,20,24</sup>

## NPs Explored: Delving into the potential of *Photorhabdus* spp.

Prior to this study different *Gammaproteobacterial* families were examined. The *Photorhabdus* genus, an *Enterobacterales* order of Gram-negative bacteria, specifically *P. laumondii* subsp. *laumondii* TTO1, was selected due to its availability and the presence of 22 BGCs (our unpublished data). We will be exploring *P. laumondii* TTO1, a model entomopathogenic bacteria with a genome size of 5.69 megabase pairs (Mbp), which carries up to 4,839 protein-coding genes, some of which have yet to be characterized<sup>1,24,25</sup>.

*Photorhabdus* is an entomopathogenic bacteria which has a fascinating and complex lifecycle<sup>26,27</sup>. A well-studied aspect of its lifecycle is its relationship with its hosts which can be separated in two forms. It is reported that these forms display distinct NP profiles and phenotypes, which could be referred to as phenotypic heterogeneity<sup>27</sup>. During its pathogenic form (P-Form), *Photorhabdus* is in the active phase which correlates with its exponential growth phase and consists of two types of host-relationships<sup>5</sup>. The first is a mutualistic symbiont relationship with *Heterorhabditis bacteriophora*<sup>5,27</sup>, a species of soil dwelling nematode. In contrast, the second relationship is infectious. *Photorhabdus* is released by the nematodes inside its prey, commonly the wax moth *Galleria mellonella* insect larvae, to kill and sterilize. This provides the bacteria and the nematodes with a food source to support their growth and reproduction<sup>5,27,28</sup>. As the nematode reproductive cycle starts, a genetic locus in *Photorhabdus* called *madswitch* (maternal adherence switch) is activated. With the *madswitch* ON, a change of form (M-Form) occurs where the bacteria stick to and invade the gut of the mother nematode. This ensures its presence in the developing generation of infective juveniles (IJ).<sup>27</sup>

The two forms are distinct. When the *madswitch* is OFF, *Photorhabdus* is in its P-Form which is necessary for the development and growth of the nematode as well as insect larvae

infection<sup>27,28</sup>. It forms large pigmented colonies which express an array of NPs, including antibiotics and crystal inclusion bodies<sup>5,26</sup>. In contrast, when the *madswitch* is ON, the bacteria switches to the M-Form; a less active phase required for its transmission to the developing IJ<sup>27</sup>. This form is reported to consist of small unpigmented colonies with no antibiotic production and no infection or growth support function.<sup>27</sup>

In our investigation of this fascinating bacteria, we have confirmed the presence in its genome of all the elements required for NP expression using transcriptional activators also known as bacterial enhancer binding proteins (bEBPs). Their presence is essential for the continuation of this study.

### **NPs production: Unraveling the Role of $\sigma^{54}$ in Transcription**

In bacteria, transcription is initiated by the activation of the sigma factors-RNA polymerase ( $\sigma$ -RNAP) holoenzyme complex<sup>16,29-31</sup>. Different sigma factors are found to regulate the transcription of different groups of BGCs, and can be separated in two classes<sup>30,31</sup>. First, the  $\sigma^{70}$  family holds housekeeping sigma factor and is responsible for the majority of transcription initiated during growth<sup>31,32</sup>. The second class is represented by sigma factors structurally related to  $\sigma^{70}$ , known as  $\sigma^{54}$ , which regulate diverse responses to environmental stimuli<sup>31-33</sup>. Both classes direct the holoenzyme complex to bind to a promoter region located at the start of the gene<sup>30,31</sup>. Each of the two classes have a conserved promoter element binding sequence<sup>31</sup>. Some members of the  $\sigma^{70}$  family, when associating with RNAP, bind to a specific consensus sequence at positions -35 (TTGACA) and -10 (TATAAT); whereas  $\sigma^{54}$  has its own conserved and function critical consensus sequence at positions -24 (GG) and -12 (TGC)<sup>31</sup>.

After binding to the promoter elements, the  $\sigma^{70}$  holoenzyme is able to initiate transcription

readily through the isomerization of the double-stranded DNA (dsDNA)<sup>31</sup>. However, the  $\sigma^{54}$  holoenzyme must enlist the assistance of a bEBP, to help it convert its initial energetically favorable closed complex to an open one<sup>31</sup>. It is this latter key feature that we explore in this study: the important role that these  $\sigma^{54}$ -dependent bEBPs have in controlling the expression of different NPs.

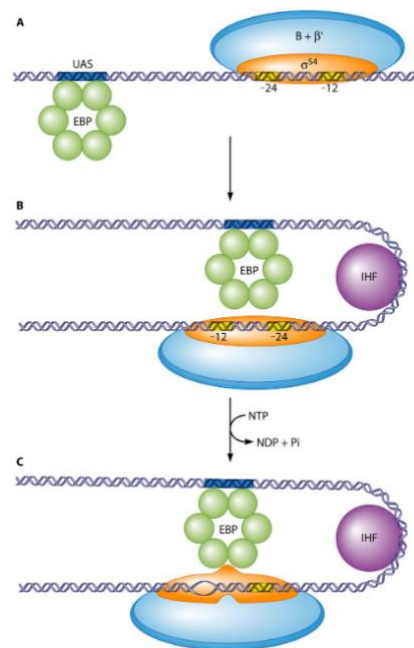
$\sigma^{54}$  has a distinct structure that is composed of three regions. Region I, found at the N-terminus, is responsible for binding to the bEBPs. Region II doesn't seem to play an essential role, its function has yet to be determined<sup>31</sup>. Finally region III located at the C-terminus, is a conserved region and is responsible for binding to the promoter element at position -24 as well as binding to the RNAP core<sup>30,31</sup>. As the holoenzyme forms,  $\sigma^{54}$  directs it to bind loosely and in a closed conformation to the conserved promoter elements at the start of the  $\sigma^{54}$ -dependent genes<sup>30,31,34</sup>. The -12 element is essential since this is the specific region where the melting of the dsDNA takes place to initiate transcription.<sup>31</sup> In the inactive form of the holoenzyme (the closed conformation),  $\sigma^{54}$  binds to the -12 elements and blocks the dsDNA from melting. It is only by interacting with a bEBP at a conserved GAFTGA motif that this inhibitory binding can be released. This interaction causes a conformational change that switches the holoenzyme into an open complex which initiate the dsDNA melting process and thus transcription.<sup>31</sup>

### **NPs Regulation: Understanding the Function of bEBPs**

As discussed, bEBPs are proteins that are required to convert the closed  $\sigma^{54}$ -RNAP holoenzyme complex into an open one<sup>30,31</sup>. Many bEBPs are composed of 3 domains; a regulatory (R) domain, a AAA+ central (C) domain and a DNA binding (D) domain. Transcriptional activation usually requires the detection by the R domain of environmental cues through a signal

transduction cascade<sup>31</sup>. This leads to phosphorylation of the R domain, ligand binding and/or protein-protein interaction, all of which will in turn induce a conformational change in the C domain that will activate the bEBP<sup>31</sup>. This latter change in the C domain will generally promote as seen in Figure 1A, bEBP hexamer self-assembly and binding through its oligomerized D domains to the upstream activator sequence (UAS or enhancer site). The interaction of  $\sigma^{54}$ -RNAP holoenzyme to the active bEBP oligomer is facilitated by DNA looping (Figure 1B). Upon ATP hydrolysis, the bEBP- $\sigma^{54}$ (-RNAP) interaction tightens, leading to a conformational change in the  $\sigma^{54}$ -RNAP and the melting of the dsDNA, thus initiating transcription (Figure 1C). This general sequence of activation can be observed with C<sub>4</sub>-dicarboxylic acid transport protein D (DctD), a well-studied bEBP.<sup>31</sup>

bEBPs in their inactive form are normally dimeric. Usually, a functional bEBP oligomer (hexamer) is composed of 3 dimers. As indicated previously each monomer is composed of three domains<sup>31</sup>. Of these, only the C domain is required for activity.<sup>35</sup>



**Figure 1. bEBP mechanism of action.**<sup>31</sup>



## **Investigating NP expression via a novel approach employing bEBPs**

Through this study, we will demonstrate that when rendered constitutively activated bEBPs endogenous to *P. laumondii* TTO1 activate NP biosynthesis. This approach bypasses the need for cues and heterologous expression, in principle greatly accelerating the discovery of new NPs. Furthermore, we will confirm, through the expression of DctD C domain (DctD<sub>(141-394)</sub>), that  $\sigma^{54}$  is an important global regulator in *P. laumondii* TTO1 responsible for the expression of numerous BGCs.

## **MATERIALS and METHODS**

### **Bioinformatics**

An AntiSMASH<sup>36</sup> analysis was performed by Dr. Brandon Findlay , under default settings, comparing to the database the genome of different Gram-negative bacterial species to identify new BGCs. These genomes were uploaded from the publicly available database, NCBI. A type strain with a high count of new BGCs was identified and used for this study.

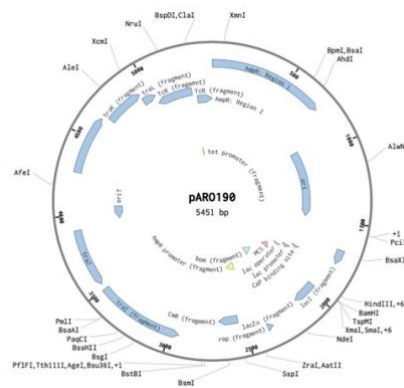
An NCBI pBLAST analysis was performed to identify bEBPs present in the selected strain's genome. The protein sequence of *Sinorhizobium meliloti* DctD core domain (DctD<sub>(141-394)</sub>) was used as a template. The bEBPs identified were used in subsequent experiments.

### **Strains and Plasmids**

All the strains and plasmids used are listed in Table 1. *Escherichia coli* MFDpir was generously donated by the Fernandez Herrero Lab (National Center for Biotechnology in Madrid). pTOX5 was purchased from AddGene (Watertown, USA). pARO190 was purchased from Cedarlane (Burlington, Canada), and Prerna Singh (PhD. candidate) confirmed its sequence in conjunction with Plasmidsaurus (Oregon, USA). The full plasmid map is shown in Figure 2.

**Table 1.** Bacterial strains and plasmids used in this study.

Strains and plasmids	Function	Source
<i>Photorhabdus laumondii</i> TTO1 (DSM15139)	Strain of interest.	Findlay lab
<i>Escherichia coli</i> MFDpir	Donor strain. Auxotrophic for diaminopimelic (DAP)	Fernandez Herrero Lab (National Center for Biotechnology in Madrid)
<i>E. coli</i> DH5 $\alpha$	Blue/White screening.	Findlay lab
pARO190	Used for conjugation. Contains the OriT element.	Cedarlane
pTA-Mob	Used for conjugation. Assists in transferring pARO190.	Findlay lab
pTOX5	Used to introduce the <i>rpoN</i> gene knockout cassette.	AddGene



**Figure 2. pARO190 map.**  
Plasmid map obtained after Plasmidsaurus sequence analysis.

## Media and Chemicals

Lysogeny broth/agar (LB, LA) and tryptic soy broth/agar (TSB, TSA) were used as growth media for the *E. coli* strains. TSB/TSA were used as growth media for *P. laumondii* DSM15139. All media were purchased from Sigma-Aldrich (Oakville, Canada).

X-gal (200  $\mu\text{g}/\text{mL}$ ) and IPTG (100  $\mu\text{M}$ ) were added to LB media for the identification of mEBPs constructs in the blue-white screening experiment. Diaminopimelic acid (DAP, 0.3 mM)

was added to LB, LA, TSB and TSA media to ensure cell growth of the MFDpir donor strain during the conjugation experiment. Carbenicillin (Crb, 100 µg/mL) was used to select for colonies hosting pARO190 recombinants. Gentamicin (Gm, 10-15 µg/mL) was used to select for colonies hosting pTA-Mob. Chloramphenicol (Cm, 100 µg/mL) was used to select for colonies hosting the pTOX5 recombinants.

IPTG (0.5 mM) was added to TSB media to induce mEBPs expression during the NPs production experiment. The chemicals for the LC-MS run are: HPLC-grade H<sub>2</sub>O, LC-MS-grade formic acid, acetone and LC-MS-grade methanol were all obtained from Fisher chemical, while HPLC-grade acetonitrile (CAN) was obtained from Sigma-Aldrich.

### **EBPs modification (mEBPs) and recombinant plasmid construction**

Primer design. All primers designed are under 40 nucleotide residues (bases). The G-C content ranges between 50-69 %, except for the bEBPs modification (mEBPs) reverse complement (RC) primers, which are under 60 b due to the His tag. The melting temperatures ( $T_m$ ) range varies between 53-61 °C. The 3' end had G and C bases added as frequently as possible.

bEBPs. Colony PCR (cPCR) was performed to confirm the presence of bEBPs in the strain of interest, using primers found in Table A1. A *P. laumondii* colony was resuspended in 50 µL of mqH<sub>2</sub>O and heated at 95 °C for 5min, then 5 µL of this liquid was used as template, with Phusion DNA polymerase. The resulting PCR products were visualized on a 1% agarose gel. The PCR settings are found in Table 4 (All reagents were from NEB and IDT DNA).

mEBPs amplicons. To implement the EBPs modifications (Table 2), a cPCR (as described above) using *P. laumondii*'s genome as DNA template was performed with the primers from Table 3. The resulting amplicons were then directly used to generate the recombinant plasmids.

Recombinant plasmid. The amplicons (mEBPs) were digested with restriction enzymes using HindIII and KpnI and ligated with T4 DNA ligase to the appropriately- digested pARO190 (except for *glrR*, where HindIII and Sall were used). All reagents used were from New England Biolabs. After an overnight (ON) incubation at 4 °C. The recombinant plasmids mix was chemically transformed into chemically competent cells (CCC) *E. coli* DH5 $\alpha$ , following a protocol from OpenWetWare<sup>63</sup> with one correction: the volume of TSS (transformation and storage solution) buffer used was 3 % of the initial incubation volume.

Blue-White colony screening. Following the chemical transformation, based on a protocol from OpenWetWare<sup>64</sup> with adjustments including incubating for 45 s at 42 °C for heat shock and 2 h at 225 rpm for growth initiation, the transformed cells were then plated on LA/X-gal/IPTG/Crb+ and incubated ON at 37 °C. Identification of the of the clones with the desired recombinant plasmids was achieved through the blue-white screening. Three white colonies per mEBP were incubated in LB/Crb+ ON at 225 rpm and 37 °C, then stored at -80 °C in 20 % glycerol. The recombinant plasmids were extracted and confirmed by sequence analysis (Genome Quebec Innovation Center/McGill University).

**Table 2.** Modified bEBPs used in this work.

<b>Name</b>	<b>Region conserved</b>	<b>Source</b>
DctD <sub>(141-394)</sub>	<b>Start from codon 141 to 394</b>	Gen9
PspF-M	1 <sup>st</sup> aa modified LEU (TTG) to MET (ATG)	This work
PLU_RS06090 <sub>(<math>\Delta</math>2-304)</sub>	Start from codon <b>305</b> to stop codon	This work
PLU_RS06090 <sub>(<math>\Delta</math>2-320)</sub>	Start from codon <b>321</b> to stop codon	This work
GlrR <sub>(<math>\Delta</math>2-122)</sub>	Start from codon <b>123</b> to stop codon	This work
GlrR <sub>(<math>\Delta</math>2-131)</sub>	Start from codon <b>132</b> to stop codon	This work
TyrR <sub>(<math>\Delta</math>2-123)</sub>	Start from codon <b>124</b> to stop codon	This work
TyrR <sub>(<math>\Delta</math>2-200)</sub>	Start from codon <b>201</b> to stop codon	This work
PrpR <sub>(<math>\Delta</math>2-208)</sub>	Start from codon <b>209</b> to stop codon	This work

For DctD<sub>(141-394)</sub> only the C domains was used, with the sequence derived from *S. meliloti*. For the rest of the modified bEBPs the R domain was deleted. These sequences were obtained from *P. laumondii* (DSM15139) via cPCR.



the palette was resuspended with TSB/DAP. The samples were mixed in a 3:1 ratio (recipient to donor), plated as a small circle on a TSA/DAP and incubated ON at 30 °C. Day2, cells were collected from the ON plate, resuspended in TSB (DAP removal selects against the donor strain, *E. coli* MFDpir) then plated on TSA/Crb+ and incubated 48-72 h at 30 °C. Three colonies per mEBP variant were incubated in TSB/Crb+ ON at 225 rpm and 30 °C, then stored at -80 °C in 20 % glycerol. The presence of the strain and plasmid was confirmed via cPCR (as described above), using M13 primers to verify the presence of pARO190, and primers targeted to *pir* and *pspF* to confirm the strain as *E. coli* or *P. laumondii*, respectively (Table 5).

**Table 5.** Primers used to confirm strains or plasmid identity.

<b>Primers</b>	<b>Sequences</b>
FW- <i>pir</i>	TCACACCCTGGCTCAACTTC
RC- <i>pir</i>	TTTGGGAGGTACGGTTTCATCA
FW- <i>pspF</i>	TGGGTGAAGCAAACAGCTTTC
RC- <i>pspF</i>	AGTCGTAGTTTCTCTGCTGCTT
FW-M13	GTAAAACGACGGCCAGT
RC-M13	CAGGAAACAGCTATGAC

All primers were ordered from IDT DNA. Abbreviations, FW: forward primer and RC: reverse complement primer

### **NP production, extraction, and analysis protocols**

NP production. A 4 day growth protocol from Maythem Ali was adjusted. An overnight culture of *P. laumondii* carrying the plasmid of interest was diluted 100x in TSB/Cr<sup>+</sup>. IPTG was then added to a final concentration of 0.5 mM, alongside 200 µL of sterile Amberlite XAD-16 beads per 10 mL of sample. Cells were then incubated at 30 °C, 250 rpm for 4 days. A sample containing all the elements except the recipient strain was used as a negative control. Each strain was tested in triplicate.

Extraction. After the 4 day incubation, the samples were centrifuged at 4 °C, 4 krpm for 5 min. The pellets were collected and resuspended with cold TBS, then centrifuged for 1min at 13.3 krpm.

The washes were repeated 3 times. The pellets were resuspended with a 1:1 MeOH to Acetone mix; incubated at 30 °C, 250 rpm for 15 min; centrifuged 1 min at 13 krpm then the supernatants were collected and speed vacuumed until obtaining pellets. Samples were stored at -80 °C.

Sample preparation for LC-MS. The samples' pellets were resuspended using MeOH (with leucine enkephalin acetate as an internal standard at 18 mM); sonicated for 10 min, then centrifuged 1 min at 13 krpm. Supernatants were then transferred to clear vials. Aliquots of 20 µL of each sample were pooled for quality control testing and a 1:1 solution of methanol: acetone was used as a blank. All samples were stored in the dark at room temperature until the LC-MS run.

LC-MS run. LC-MS/MS analyses were performed on an Agilent 1100 LC system coupled to a Thermo LTQ Orbitrap Velos mass spectrometer equipped with a heated electrospray ion source at positive mode. The column used was a CORTECS ® T3 2.7 µm, 2.1x100mm column at a flow rate of 0.3 ml min<sup>-1</sup> with a 10 µl injection volume. The compounds were eluted using a 34 min gradient at a flow rate of 300 µL/min with mobile phase A (water containing 0.1 % FA) and B (ACN containing 0.1 % FA). The gradient started at 5 % B and held for 1 min, linear gradients were achieved to 95 % B at 20 min (Table 6). The mobile phase was held at 95 % B for 7 min, then reduced to 5 % B for 7 min. A full MS spectrum ( $m/z$  150-2000) was acquired in the Orbitrap at a resolution of 100000, with the five most abundant singly and doubly charged ions each second selected for MS/MS fragmentation in the linear trap, with the option of dynamic exclusion. Compound fragmentation was performed using a collision induced dissociation at normalized collision energy of 35 % with activation time of 10 ms. The spectra were internally calibrated using diisooctyl phthalate ( $m/z$  391.2843 Da) as a lock mass.

Data analysis: Compound discoverer 3.3 and Thermo XCalibur Qual Browser were used to analyse the data collected from the LC-MS run. The data were internally adjusted using a



compound present in the background as a reference ( $m/z$  245.1286 Da) to account for a shift in mass, as determined by the quality control observation. This was followed by filters based on a mass tolerance of 5ppm at MS1 and the presence of an MS2 fragmentation profile with a mass tolerance of 0.6Da.

**Table 6.** PCR cycle used in this study.

<b>Time (min)</b>	<b>A (%)</b>	<b>B (%)</b>	<b>Flow (mL/min)</b>	<b>Max. Pressure Limit (bar)</b>
0.00	95.0	5.0	0.300	340.00
1.00	95.0	5.0	0.300	340.00
20	5.0	95.0	0.300	340.00
27.00	5.0	95.0	0.300	340.00
27.00	95.0	5.0	0.300	340.00
34.00	95.0	5.0	0.300	340.00

### **Knockout Cassettes**

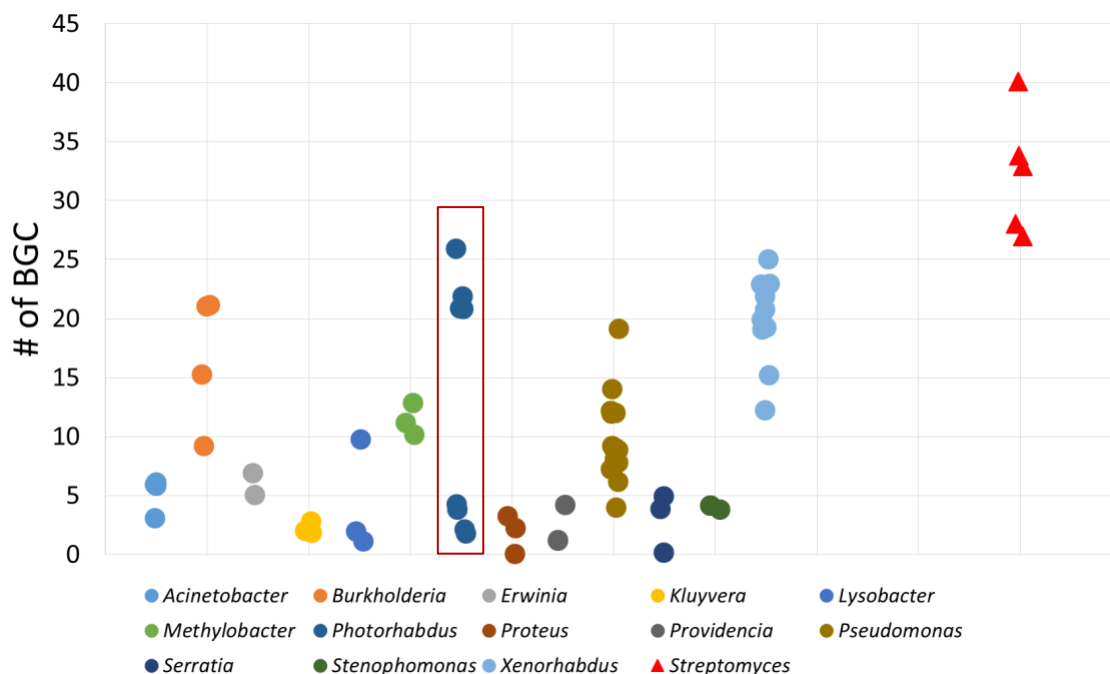
Knockout cassettes for five genes of interest in *P. laumondii* were designed and ordered from IDT DNA, including *rpoN* (Table A2). They were then cloned independently into pTOX5 (Figure A1 and Table A3) using T4 DNA ligase at restriction sites EcoRV and PacI. All reagents used were from New England Biolabs.

## RESULTS

### Bioinformatic analysis

#### 1. Strain selection.

To evaluate the best strain for this study, Dr. Brandon Findlay compared 13 different families of *Gammaproteobacteria*. The genomes were obtained from NCBI and analysed using antiSMASH<sup>36</sup> under default settings. Several strains of *Streptomyces* spp. were added for scale (Figure 3). Most families analysed had less than 15 BGCs, while only 4 families were found to have strains with more than 19 BGCs. Among them, the *Photorhabdus* spp. (Figure 3, red box) showed great potential. Due to it being a type strain and the presence of 22 newly identified BGCs (Table A4), the *Photorhabdus laumondii* DSM 15139 strain was selected to serve as the model organism in this work.



**Figure 3. BGCs' distribution amongst *Gammaproteobacterial* families.**

*Photorhabdus* spp. was selected (red box) as a model Gram-negative entomopathogenic bacterium due to its potential for BGCs count. *Streptomyces* spp. was added for scale. Unpublished data. Figure made by Dr. Brandon Findlay.

## 2. bEBPs identified in *P. laumondii* TTO1.

To identify the  $\sigma^{54}$ -bEBPs present in *P. laumondii* TTO1, we used as a template the *Sinorhizobium meliloti* DctD core (C) domain (DctD<sub>(141-394)</sub>), containing the  $\sigma^{54}$  binding domain<sup>35</sup>, obtained from Gen9. The NCBI protein-protein BLAST (blastp) search was conducted under default settings, revealing a total of six bEBPs with E values below a cut-off of 1e-10: GlnR (3e-73), GlnG (3e-61), TyrR (3e-57), PspF (1e-56), PrpR (3e-52) and PLU\_RS06090 (2e-59) (Table 7 and Figure A2).

**Table 7.** Identified  $\sigma^{54}$ -dependent bEBPs in *P. laumondii* TTO1.

Genes	Description	E value	Old locus tag	Accession
<i>glnR</i>	two-component system response regulator	3 e-73	plu3311	WP_011147503.1
<i>glnG</i>	Nitrogen regulatory protein RN(I)	3 e-61	plu0235	WP_011144634.1
<i>tyrR</i>	Transcriptional regulatory protein TyrR	3 e-57	plu2580	WP_011146802.1
<i>pspF</i>	Phage shock protein operon transcriptional activator	1 e-56	plu2586	WP_011146808.1
<i>prpR</i>	Propionate catabolism operon regulatory protein PrpR	3 e-52	plu3543	WP_011147723.1
<i>PLU_RS06090</i>	NRPS – Unknown	2 e-59	plu1233	WP_011145558.1

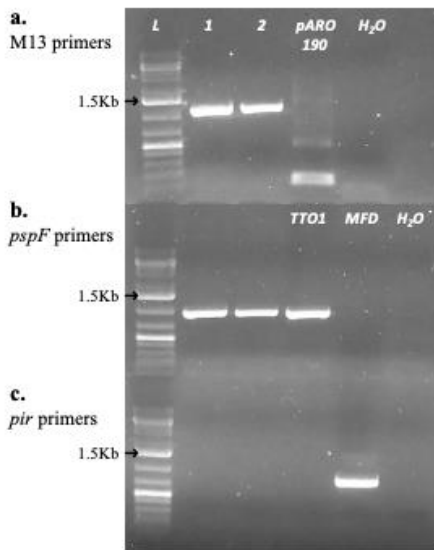
Using NCBI BLASTp and NCBI database. bEBPs identified in *P. laumondii* TTO1 using  $\sigma^{54}$  binding sequence (core domain) from *S. meliloti* DctD bEBP as a template.

Further enquiry revealed that among these six putative bEBPs, the GAFTGA sequence motif was present in four, namely GlnR, GlnG, PspF and PLU\_RS06090. This motif is necessary for the bEBP- $\sigma^{54}$  interaction and thus the activation of the RNAP- $\sigma^{54}$  holoenzyme<sup>31</sup>. Despite lacking this motif, TyrR and PrpR were included in this study due to their high homology to DctD C domain, as indicated by their respective E value 3e-57 and 3e-52, aiming to assess their effect on NP biosynthesis in *P. laumondii*.

## Generating variants

The presence of all of the bEBPs in *P. laumondii* TTO1, save for *glnG*, was then verified by cPCR in *P. laumondii* TTO1 using primers from Table A1. These five bEBPs were then modified by cPCR using primers from Table 3 to remove the leading regulatory (R) domain as indicated in Table 2, and referred to hereafter as mEBPs. Different deletion ranges were explored for some bEBPs based on a UniProt review of their R domain (Table A5). Those with unidentified R domains like PrpR had all the region preceding the core domain deleted, whereas those with known regulatory elements underwent two deletions: one removing only the regulatory elements, and the second deleting the entire region before the core domain. For PLU\_RS06090, GlrR and TyrR, two deletions were obtained for each: PLU\_RS06090<sub>(Δ2-304)</sub> and PLU\_RS06090<sub>(Δ2-320)</sub>; GlrR<sub>(Δ2-122)</sub> and GlrR<sub>(Δ2-131)</sub>; as well as TyrR<sub>(Δ2-123)</sub> and TyrR<sub>(Δ2-200)</sub>. For PrpR as mentioned only one deletion was obtained PrpR<sub>(Δ2-208)</sub>. As for PspF, since it did not contain an R domain, a modification to its first codon (TTG, Leu) to a start codon (ATG, Met) was the only change required. These mEBPs including DctD<sub>(141-394)</sub>, were then ligated into pARO190, identified in *Escherichia coli* DH5<sub>α</sub> using blue-white screening, sequenced to confirm the modifications, and then chemically transformed into donor strain *E. coli* MFDpir. With the exception of PrpR<sub>(Δ2-208)</sub>, all of these mEBPs were then successfully conjugated into *P. laumondii* TTO1, thus generating the different variants named after the mEBP they carry.

Before successfully introducing the recombinant plasmids in *P. laumondii* TTO1, multiple protocols were attempted, over 100 chemical transformations, 30 electroporation and 27 conjugations, all yielding no results. The first successful conjugation was that of TyrR<sub>(Δ2-200)</sub> using an optimized protocol from the Bode lab. The presence of the different mEBPs in *P. laumondii* TTO1 was confirmed via cPCR using primers from Table 5, as demonstrated in Figure 4.



**Figure 4. cPCR confirmation of pARO190-tyrR<sub>(Δ2-123)</sub> conjugation.** For each gel: column 1 is the ladder (L); column 2 is cPCR product from TyrR<sub>(Δ2-123)</sub>Y variant (1); column 3 is cPCR product from TyrR<sub>(Δ2-123)</sub>B variant (2); all the remaining columns are identified with the different controls. **a.** Verifying plasmid presence using M13 primers; **b.** Validating *P. laumondii* TTO1 strain presence using pspF primers; **c.** Confirming MFD(pir<sup>+</sup>) strain absence using pir primers. The verification primer sequences are found in Table 5.

### Effect of mEBPs on Cell Growth

Upon the successful generation of the variants containing the different mEBPs, triplicates of each variant were produced, and the three main phenotypes of color, shape, and growth rate of each sample were recorded (Table 8). Singlicate samples of the media and *P. laumondii* TTO1 strain were assessed as controls.

All variants exhibited normal growth, with the exception of TyrR<sub>(Δ2-200)</sub>. This variant had notably slower growth than the other samples, forming very small, bright yellow colonies. The variants PLU\_RS06090<sub>(Δ2-320)</sub>, GlrR<sub>(Δ2-131)</sub> and TyrR<sub>(Δ2-123)</sub> grew at normal rates, but formed a mixture of beige and yellow-pigmented colonies, suggesting heterogeneous gene expression. To distinguish between the two different colony types, subgrouping by letters was implemented, those with yellow or beige pigmentation were designated Y or B, respectively (Table 8). The complete observations on the samples phenotypes and metabolites extracts can be found in Table A6.

**Table 8.** Variants phenotypic categorization.

Categories	Yellow colonies	Beige colonies
<b>Round shaped and regular growth</b>	PLU_RS06090( $\Delta$ 2-320)Y; GlrR ( $\Delta$ 2-131)Y; TyrR( $\Delta$ 2-123)Y	TTO1 WT; pARO190; DctD <sub>(141-394)</sub> ; PspF-M; PLU_RS06090( $\Delta$ 2-304); PLU_RS06090( $\Delta$ 2-320)B; GlrR( $\Delta$ 2-122); GlrR( $\Delta$ 2-131)B; TyrR( $\Delta$ 2-123)B
<b>Small and slow growth</b>	TyrR( $\Delta$ 2-200)	-

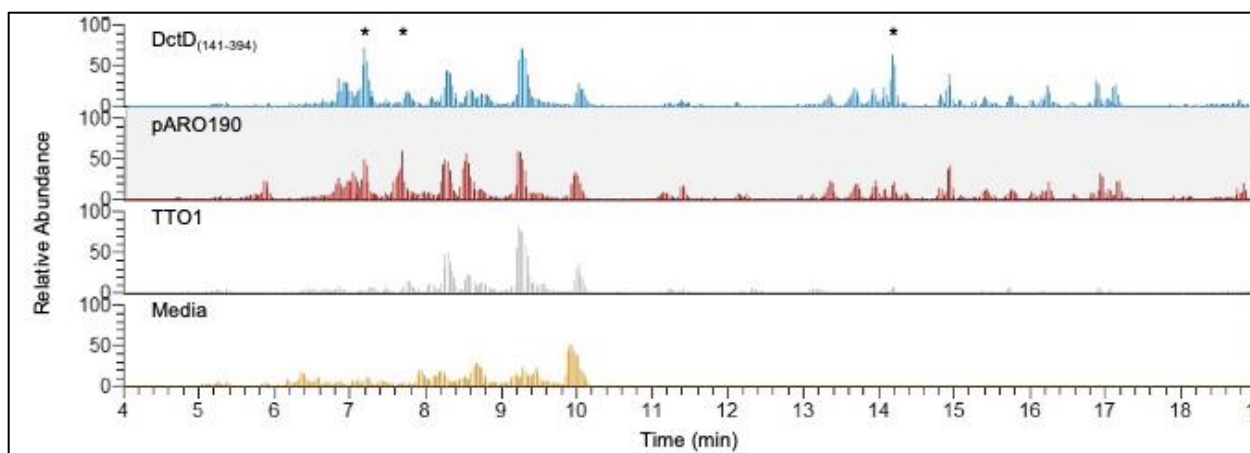
Each variant was named based on the mEBP it carries. Y (yellow) and B (beige) reflect the subgrouping for the same mEBP.

### Assessing the effect of DctD<sub>(141-394)</sub> on NP biosynthesis

#### 1. Effect on metabolite expression.

To be able to analyse the effect that the different mEBPs have on *P. laumondii* TTO1 NP expression, this approach had to be tested using the well-studied bEBP, DctD. To document the effect of DctD<sub>(141-394)</sub>, an LC-MS analysis was performed with the help of Heng Jiang, a mass spectrometry specialist at Concordia University. The prepared LC-MS samples were run in positive mode and a chromatogram, truncated due to background noise (Figure A2), showing the DctD<sub>(141-394)</sub> variant metabolite profile (blue) was obtained as seen in Figure 5. Media, *P. laumondii* TTO1 and *P. laumondii*-pARO190 samples were used as controls.

In the *P. laumondii* TTO1 strain chromatogram (gray), metabolites expression was located between 7 and 11 min, with moderate peak intensity. For the *P. laumondii*-pARO190 strain chromatogram (red), we noted significant new metabolite expression in the 11-19 min window. Sequencing of the pARO190 showed no unexpected genetic material present (Figure 2), suggesting that pARO190 alone is sufficient to induce a wide range of metabolite biosynthesis. Analysis of the DctD<sub>(141-394)</sub> variant revealed an increase in peaks intensity, as well as the presence of new peaks when compared to *P. laumondii*-pARO190 (asterisks). This indicates an activation in metabolite expression.



**Figure 5. Effect of DctD<sub>(141-394)</sub> on metabolites expression in *P. laumondii* TTO1.**

The chromatograms Y axis indicates the metabolites relative abundance (PH) at a scale of **8.00 E8**. The X axis indicates the elution time in minutes of the different metabolites, between 4-19 min. Asterisks denote new and high intensity peaks. Data analysed on Thermo XCalibur Qual Browser.

## 2. Effect on NP expression.

### a. Compound analysed.

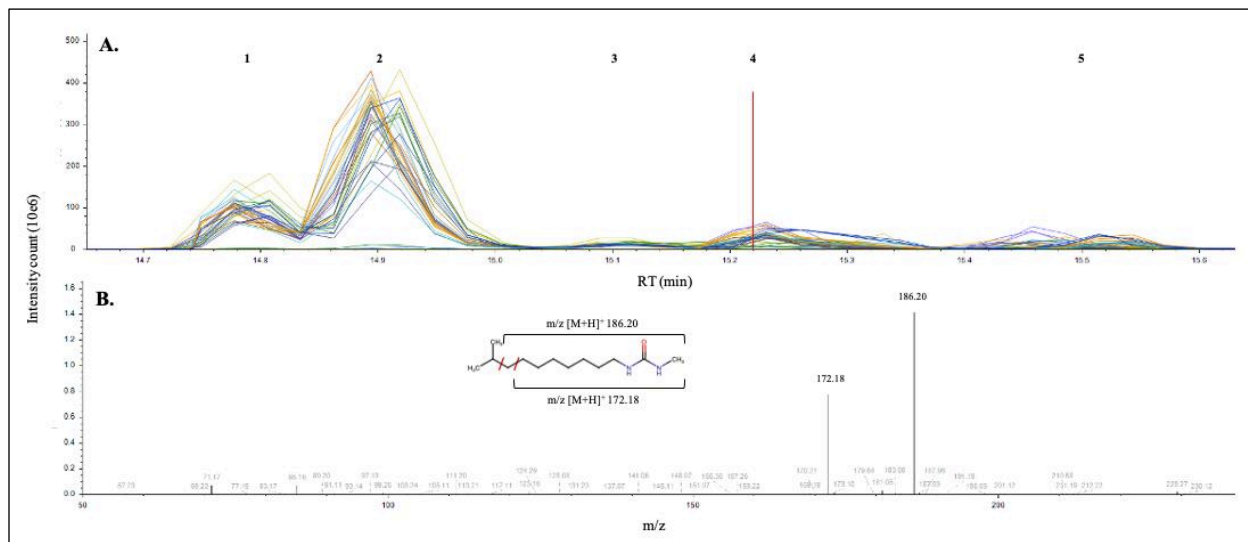
To test the induction of NP expression in *P. laumondii*, six known compounds were selected to be identified and assessed (Table 9). Two of these compounds, mevalagmapeptide A and gameXpeptide A, have proposed structures but have yet to be fully characterized<sup>24</sup>. The remaining four compounds were selected based on the fact that their structure and function are well-documented.

**Table 9.** Compounds to be analysed in this study.

Compounds	Abbreviations	Functions
Mevalagmapeptide A	MVAP-A	Unknown biological activity. <sup>21</sup>
Phurealipid A	PL-A	Act as an immunosuppressant and inhibits the development of the insect larva. <sup>38</sup>
Photopyrone D	PPY-D	Cell to cell communication (Quorum sensing) and regulation of NPs production. <sup>39,40</sup>
Isopropylstilbene	IPS	Has insecticidal and antimicrobial activities. <sup>41,42</sup>
Antraquinone 270	AQ-270	Is a pigment with some antimicrobial activity and act as a bird and insect deterrent. <sup>7</sup>
GameXpeptide A	GXP-A	Unknown biological activity. Detected only inside the host insect. <sup>41,43</sup>

Each of these compounds was identified by MS/MS in the LC-MS data using Compound Discoverer 3.3. Three out of the six compounds exhibited multiple peaks with the same MS reading, which were spread across the range of elution time, suggesting the presence of isoforms (Figure 6-8). A single peak per compound was selected for further analysis based on the presence of an MS/MS data and clear fragmentation patterns to identify the structure.

**Phurialipid A (PL-A.)** has a molecular weight of 228.2202 Da and  $m/z$  229.2274  $[M+H]^+$ . We can observe from the chromatogram in Figure 6A that a compound with a corresponding  $m/z$  has eluted at five different time points. The compound identity at position 4 (RT 15.2 min) was confirmed based on the fragments observed on the MS/MS spectrum (Figure 6B). The  $m/z$  of the two main fragments are 172.18 and 186.20, which correspond to  $[C_{13}H_{29}N_2O]^+ - [C_4H_9]$  and  $[C_{13}H_{29}N_2O]^+ - [C_3H_7]$  respectively.

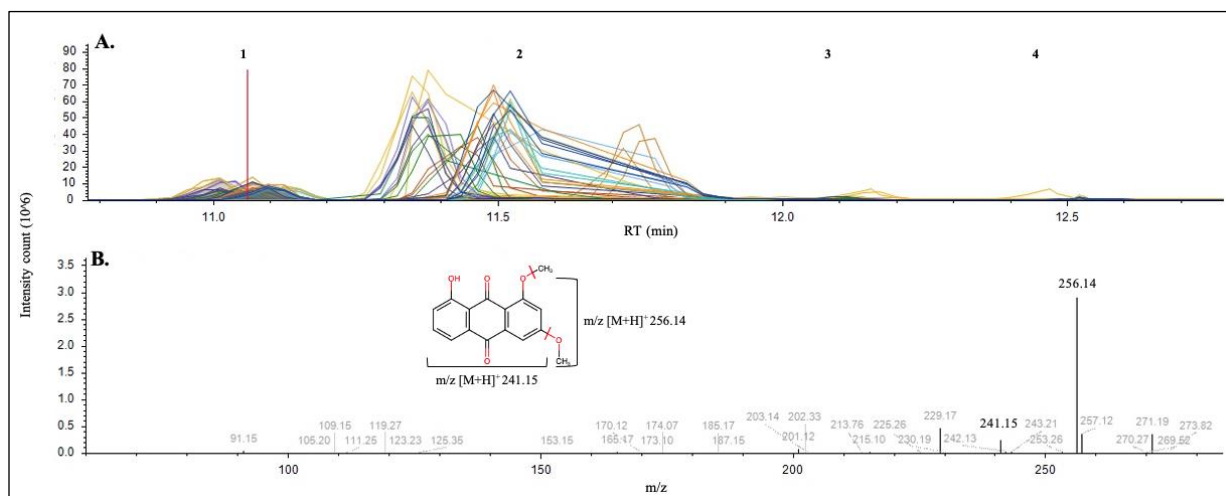


**Figure 6. PL-A identification.**

The Y axis represent the intensity count. **A.** This chromatogram reflects the data from all the variants in triplicates (39 samples). Peaks 1-5 represent the different isoforms. The red line indicates the peaks for which MS/MS fragmentation analysis was made. X axis is retention time (RT, min). **B.** the MS/MS spectrum shows the fragments obtained at RT 15.22 min. X axis is  $m/z$ . The structure of the compound with a proposed cut position for the analyzed fragments,  $m/z$  172.18 and 186.20. LC-MS data analysed on Compound Discoverer 3.3.



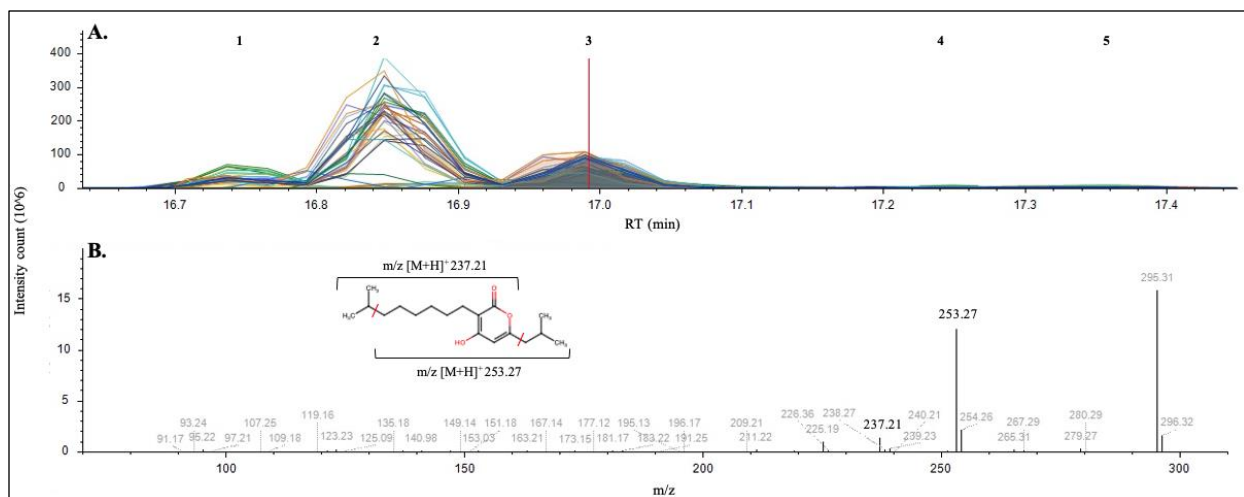
**Anthraquinone 270 (AQ-270)** has a molecular weight of 270.0528Da and  $m/z$  271.0601  $[M+H]^+$ . We can observe from the chromatogram in Figure 7A that a compound with a corresponding  $m/z$  has eluted at four different time points. The compound identity at position 1 (RT 11.0 min) was confirmed through the analysis of two fragments observed on MS/MS fragmentation spectrum. They had  $m/z$  of 241.15 and 256.14 which correspond to  $[C_{15}H_{11}O_5]^+ - [OCH_3]$  and  $[C_{15}H_{11}O_5]^+ - [CH_3]$  respectively (Figure 7B).



**Figure 7. AQ-270 identification.**

The Y axis represent the intensity count. **A.** This chromatogram reflects the data from all the variants in triplicates (39 samples). Peaks 1-4 represent the different isoforms. The red line indicates the peaks for which the MS/MS fragmentation analysis was made. X axis is retention time (RT, min). **B.** the MS/MS spectrum shows the fragments obtained at RT 11.0 min. X axis is  $m/z$ . The structure of the compound with a proposed cut position for the analyzed fragments,  $m/z$  241.15 and 256.14. LC-MS data analysed on Compound Discoverer 3.3.

**Photopyrone D (PPY-D.)** has a molecular weight of 294.2195 Da and  $m/z$  295.2268  $[M+H]^+$ . We can observe from the chromatogram in Figure 8A that a compound with a corresponding  $m/z$  has eluted at five different time points. The compound identity at position 3 (RT 17.0 min) was confirmed through the analysis of two fragments observed on MS/MS fragmentation spectrum. They had  $m/z$  of 237.8 and 253.2 which correspond to  $[C_{18}H_{31}O_3]^+ - [C_4H_9]$  and  $[C_{18}H_{31}O_3]^+ - [C_3H_6]$  respectively (Figure 8B).

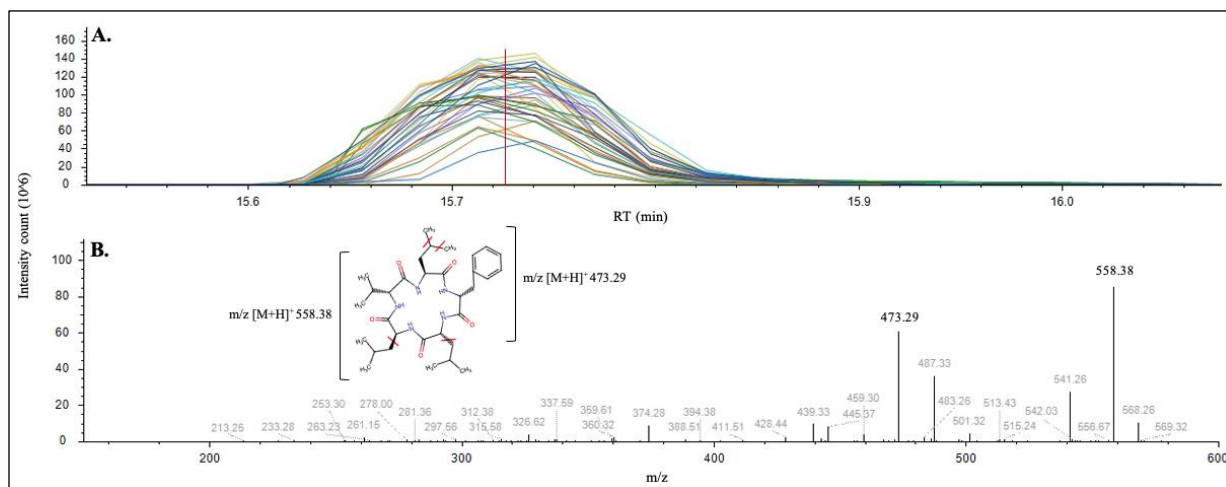


**Figure 8. PPY-D identification.**

The Y axis represent the intensity count. **A.** This chromatogram reflects the data from all the variants in triplicates (39 samples). Peaks 1-5 represent the different isoforms. The red line indicates the peaks for which the MS/MS fragmentation analysis was made. X axis is retention time (RT, min). **B.** the MS/MS spectrum shows the fragments obtained at RT 17.0 min. X axis is m/z. The structure of the compound with a proposed cut position for the analyzed fragments, m/z 237.21 and 253.27. LC-MS data analysed on Compound Discoverer 3.3.

**GameXpeptide A (GXP-A)** has a molecular weight of 585.3890 Da and m/z 586.3963 [M+H]<sup>+</sup>.

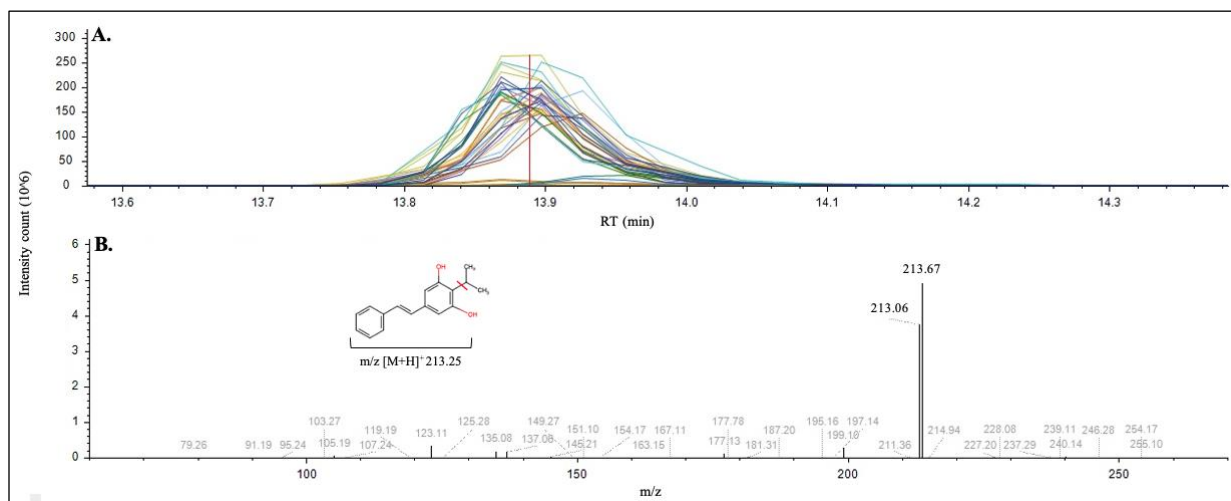
We can observe from the chromatogram in Figure 9A that a compound with a corresponding m/z has eluted once. The compound identity at position RT 15.7 min was confirmed through the analysis of two fragments observed on MS/MS fragmentation spectrum. They had m/z of 473.4 and 558.4 which correspond to [C<sub>32</sub>H<sub>52</sub>N<sub>5</sub>O<sub>5</sub>]<sup>+</sup>-2[C<sub>4</sub>H<sub>9</sub>] and [C<sub>32</sub>H<sub>52</sub>N<sub>5</sub>O<sub>5</sub>]<sup>+</sup>-2[CH<sub>2</sub>] respectively (Figure 9B).



**Figure 9. GXP-A identification.**

The Y axis represent the intensity count. **A.** This chromatogram reflects the data from all the variants in triplicates (39 samples). The red line indicates the peaks for which the MS/MS fragmentation analysis was made. X axis is retention time (RT, min). **B.** the MS/MS spectrum shows the fragments obtained at RT 15.7 min. X axis is m/z. The structure of the compound with a proposed cut position for the analyzed fragments, m/z 473.29 and 558.38. LC-MS data analysed on Compound Discoverer 3.3.

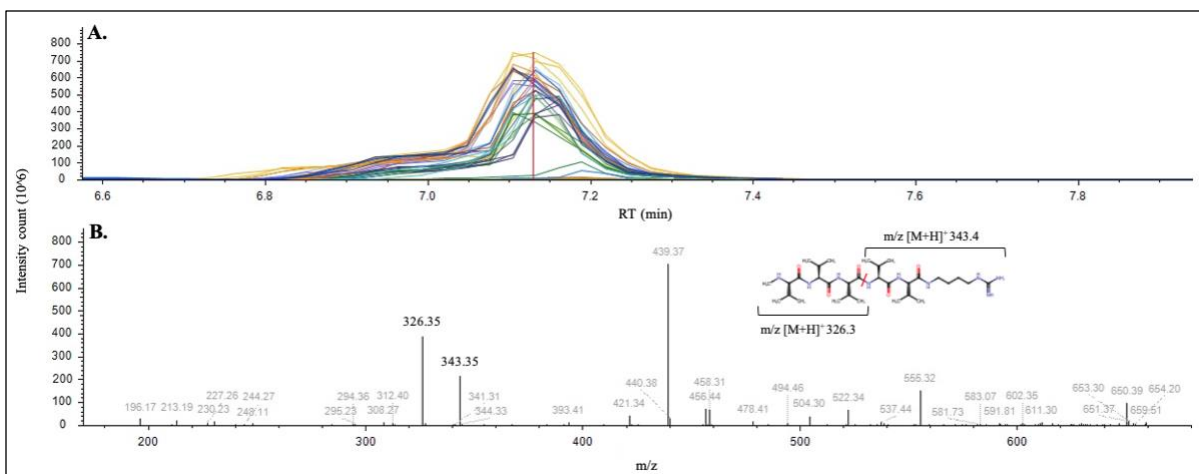
**Isopropylstilbene (IPS)** has a molecular weight of 254.1307 Da and m/z 255.1379 [M+H]<sup>+</sup>. We can observe from the chromatogram in Figure 10A that a compound with a corresponding m/z has eluted once. The compound identity at position RT 13.9 min was confirmed through the analysis of the main fragment observed on MS/MS fragmentation spectrum. It had m/z of 213.43 which corresponds to [C<sub>17</sub>H<sub>19</sub>O<sub>2</sub>]<sup>+</sup>-[C<sub>3</sub>H<sub>6</sub>] (Figure 10B).



**Figure 10. IPS identification.**

The Y axis represent the intensity count. **A.** This chromatogram reflects the data from all the variants in triplicates (39 samples). The red line indicates the peaks for which the MS/MS fragmentation analysis was made. X axis is retention time (RT, min). **B.** the MS/MS spectrum shows the fragments obtained at RT 13.9 min. X axis is m/z. The structure of the compound with a proposed cut position for the analyzed fragment, m/z 213.67. LC-MS data analysed on Compound Discoverer 3.3.

**Mevalgmapeptide A (MVAP-A)** has a molecular weight of 667.5109 Da and m/z 334.7627 [M+2H]<sup>2+</sup>. We can observe from the chromatogram in Figure 11A that a compound with a corresponding m/z has eluted once. The MS/MS spectrum at position RT 7.2 min (Figure 11B) show two main fragments with m/z 326.32 and 343.34. These two fragments correspond to the compound being split in two (343.3 + 326.3 = 2\*334.8) consistent with MVAP-A.

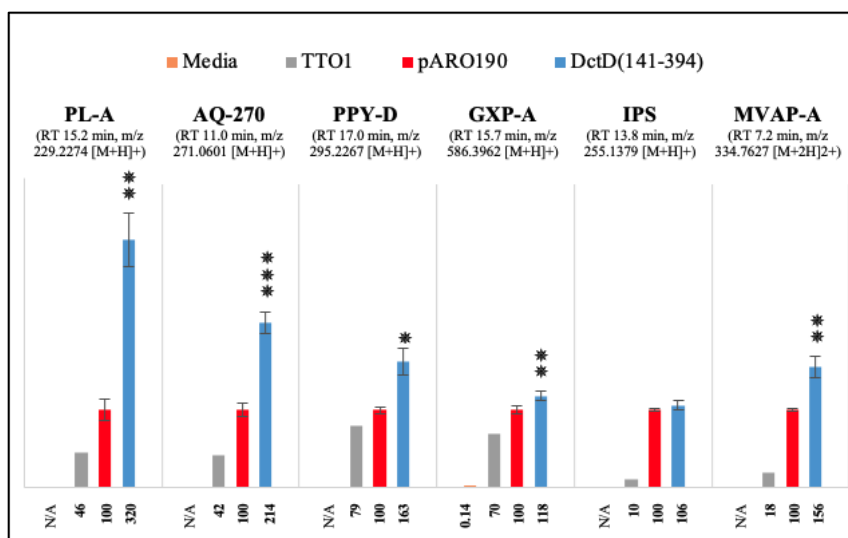


**Figure 11. MVAP-A identification.**

The Y axis represent the intensity count. **A.** This chromatogram reflects the data from all the variants in triplicates (39 samples). The red line indicates the peaks for which the MS/MS fragmentation analysis was made. X axis is retention time (RT, min). **B.** the MS/MS spectrum shows the fragments obtained at RT 7.2 min. X axis is m/z. The structure of the compound with a proposed cut position for the analyzed fragments, m/z 326.35 and 343.35. LC-MS data analysed on Compound Discoverer 3.3.

#### *b. Compound expression.*

The data collected for each of the six compounds from the DctD<sub>(141-394)</sub> variant and *P. laumondii*-pARO190 was derived from three biological replicates, while those from *P. laumondii* TTO1 and the media were analyzed in singlicate. The results of this analysis, which were normalized to *P. laumondii*-pARO190, can be seen in Figure 12. As expected, the media sample showed no significant presence of the compounds analysed, with all values below the detection limit, except for GXP-A that was detected at 0.14 %-fold change. The *P. laumondii* TTO1 strain showed readings that were systematically below those of *P. laumondii*-pARO190, indicating that the presence of pARO190 induced NP biosynthesis. In the presence of DctD<sub>(141-394)</sub>, 5 of the 6 compounds showed a statistically significant increase relative to the empty vector, with the highest expression (320%) for PL-A. No statistically significant difference was observed for the production of IPS. All raw data linked to compound production levels per variant is found in Table A7.



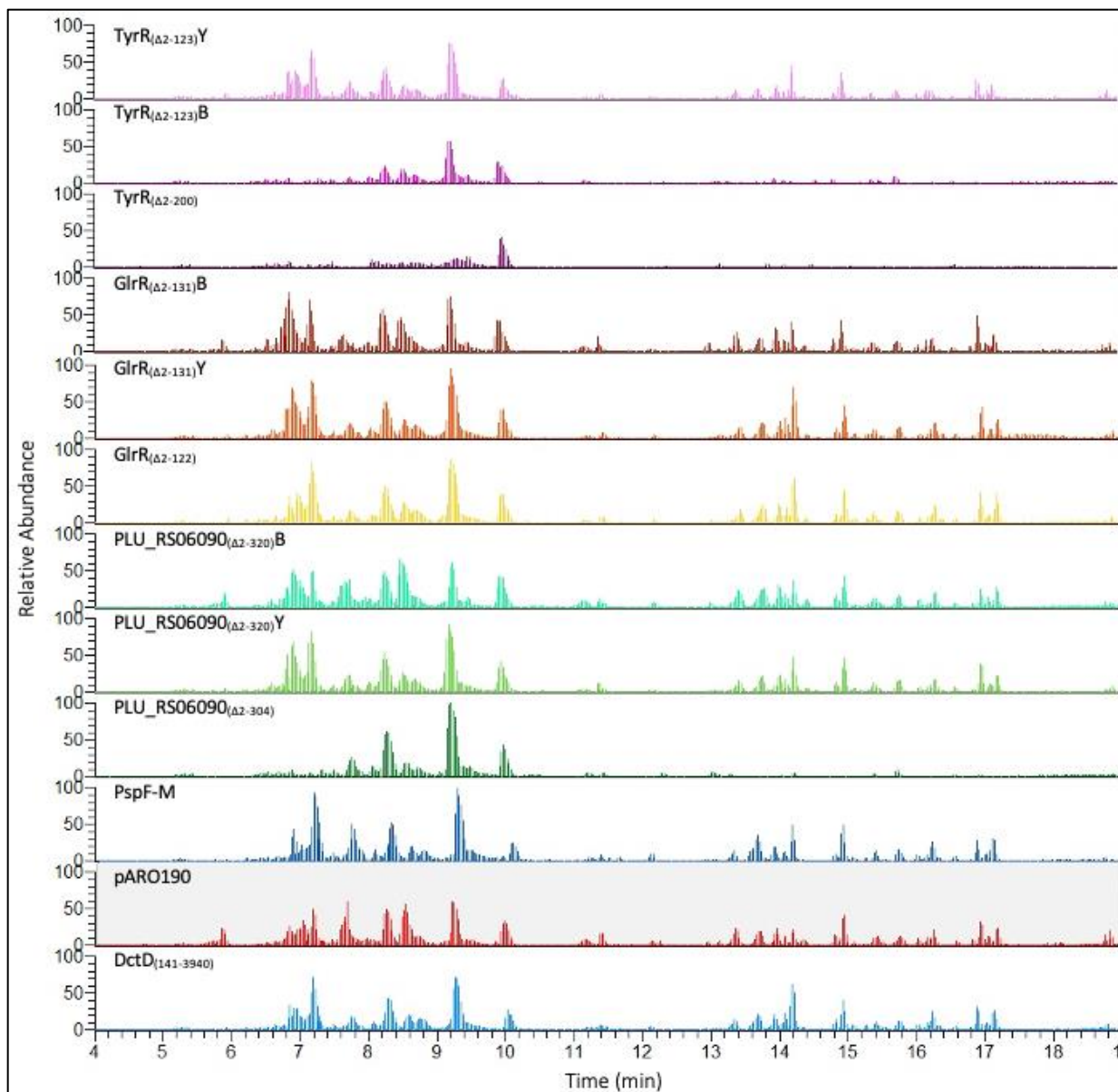
**Figure 12. Effect of DctD<sub>(141-394)</sub> on NPs expression levels in *P. laumondii* TTO1.**

The media, TTO1 and pARO190 samples are used as controls to evaluate the DctD<sub>(141-394)</sub> effects. *P. laumondii*-pARO190 expression levels are considered the normal levels in this study. The Y axis indicates the % fold change relative to the *P. laumondii*-pARO190 levels and they are displayed on the X axis per sample. The asterisks signify that the values calculated using the student t-test, were statistically significant ( $p \leq 0.05$ ). The % fold change was computed by averaging the triplicate values of relative abundance for each variant (Table A7). The compounds full name can be found in the List of Abbreviations.

## Assessing the effect of mEBPs on NP biosynthesis

### 1. Effect on metabolite expression.

With the effect of DctD<sub>(141-394)</sub> established, we turn to the mEBPs. Each mEBP was assessed in triplicate, with Thermo XCalibur Qual Browser once more used to establish the metabolite expression profile (Figure 13). Each variant shows a unique metabolite profile, with different relative abundance levels even within the same subgroup. While greater peak intensity was observed following mEBP expression in general, PLU\_RS06090<sub>(Δ2-304)</sub>, TyrR<sub>(Δ2-123)</sub>B and TyrR<sub>(Δ2-200)</sub> all show a notable reduction in signal intensity. TyrR<sub>(Δ2-200)</sub> in particular nearly completely repressed expression, giving results most similar to the media control (Figure 5).



**Figure 13. Effects of mEBPs on metabolite expression in *P. laumondii* TTO1.**

The chromatograms Y axis indicates the metabolites relative abundance (PH) at a scale of 8.00 E8. The X axis indicates the elution time in minutes of the different metabolites, between 4-19 min. The *P. laumondii*-pARO190 (red) and DctD<sub>(141-394)</sub> (light blue) variant are used as controls to evaluate the mEBPs effects. One chromatogram figure per variant triplicate was selected for display. LC-MS data analysed on Thermo XCalibur Qual Browser.

## 2. Effect on NP expression.

As previously observed, DctD<sub>(141-394)</sub> has an activating effect on the expression of all six compounds analysed. By using the DctD<sub>(141-394)</sub> variant and *P. laumondii*-pARO190 as scaling controls, representing the state of activated and basal levels of expression respectively, we are able to compare, contrast and determine the effect that each bEBP has on the expression of these compounds. The data shown in Figure 14, was compiled from three replicates which was normalized to *P. laumondii*-pARO190.

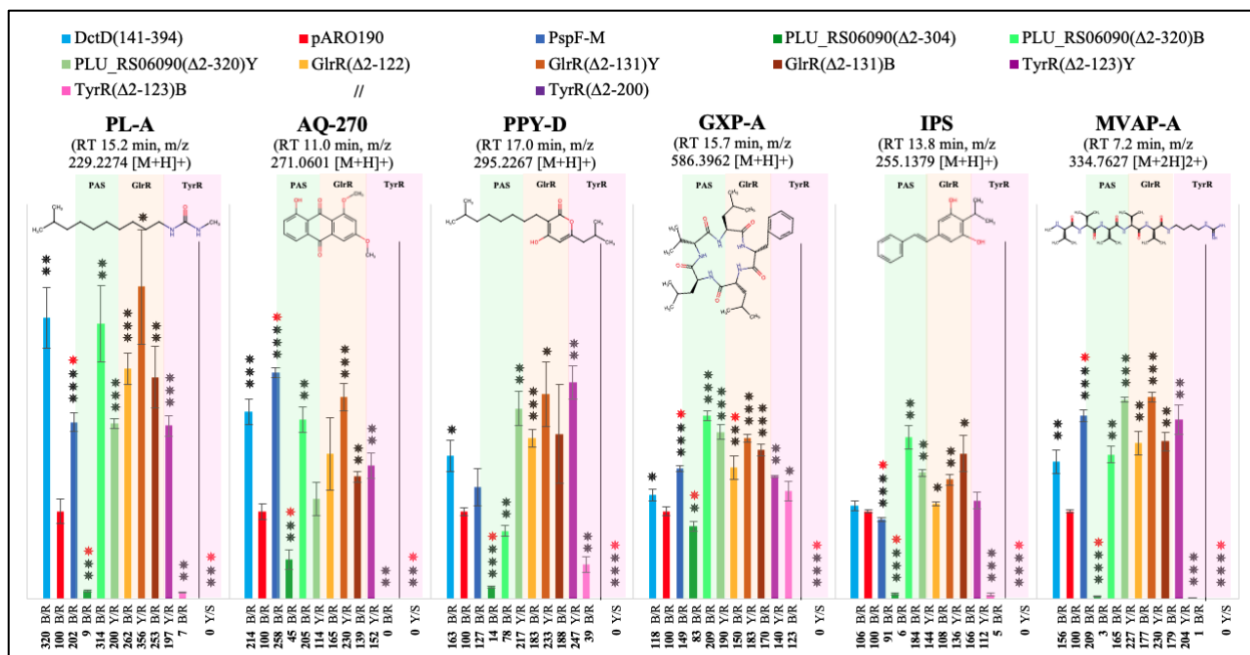
As anticipated from the metabolite profile, the TyrR<sub>(Δ2-200)</sub> variant showed no compound expression. All traces of the compounds are below the detectable limits of the LC-MS device. These results indicate a clear repressive function of the TyrR<sub>(Δ2-200)</sub> mEBP in the *P. laumondii* TTO1 strain. On the other hand, TyrR<sub>(Δ2-123)</sub>Y and B showed contradictory readings of activation and repression respectively (Figure 14). Due to this unexplained inconsistency, and the lack of a GAFTGA sequence, both TyrR<sub>(Δ2-123)</sub> variants were excluded pending further analysis.

In general, all GlrR variants activated NP biosynthesis above that of *P. laumondii*-pARO190, and in some cases higher than those of DctD<sub>(141-394)</sub>. The results obtained for the two GlrR<sub>(Δ2-131)</sub> variants showed the same general activation trend but varied greatly in the levels of compound expression. These variations require further examination to determine the cause(s), for this reason they were not be further analysed. In the case of GlrR<sub>(Δ2-122)</sub> variant, we can clearly confirm an activating function since 5 of the 6 compounds are statistically significantly higher than *P. laumondii*-pARO190 values (black asterisk). In the case of GPX-A it was above that of DctD<sub>(141-394)</sub> (red asterisk), confirming that GlrR bEBP has a general activating effect on NP expression in the TTO1 strain.

For the PLU\_RS06090 variants, the results obtained from the two PLU\_RS06090 deletions are opposite. For PLU\_RS06090( $\Delta$ 2-304) variant, all the results obtained are below those of *P. laumondii*-pARO190 but above those observed for TyrR( $\Delta$ 2-200), indicating a more moderate repressive function. For the PLU\_RS06090( $\Delta$ 2-320) variants, although their compound expression levels show great variability, they exhibited a general activation trend, with values above that of *P. laumondii*-pARO190. These observations seem to indicate that PLU\_RS06090 bEBP has two functions. Activation and repression of NP expression in the *P. Laumondii* TTO1 strain seem to depend on the extent of R domain deletion.

For the PspF-M variant, almost all the results obtained are statistically significantly above those of *P. laumondii*-pARO190. Notably, the expression levels of AQ-270, GXP-A and MVAP-A are above those seen in DctD<sub>(141-394)</sub> (red asterisk). These results indicate that PspF bEBP has a clear activating function of NP expression in the TTO1 strain.





**Figure 14. Effect of mEBPs on NP expression levels in *P. laumondii* TTO1.**

Each mEBP is represented by a colour category. The *P. laumondii*-pARO190 (red) strain and DctD<sub>(141-394)</sub> (light blue) variant are used as controls to evaluate the different mEBPs effect. In this study, *P. laumondii*-pARO190 expression levels are considered the normal levels and DctD<sub>(141-394)</sub> expression levels represent a state of activation. The Y axis indicates the % fold change relative to the pARO190 levels and they are displayed on the X axis. The phenotypic characteristics of each variant are presented as acronyms on the X axis (B/R: Beige/Regular growth, Y/R: Yellow/Regular growth and Y/S: Yellow/Slow growth). For each compound, the RT, m/z, ionic state, and chemical structure were added. The asterisks signify that the values calculated using the student t-test were statistically significant ( $p \leq 0.05$ ) (black relative to pARO190 and red relative to DctD<sub>(141-394)</sub>). The % fold change was computed by averaging the triplicate values of relative abundance for each variant (Table A7). The compounds' full names can be found in the List of Abbreviations.

## **DISCUSSION**

In this study, we aimed to verify if  $\sigma^{54}$ -dependent bEBPs could function as regulators of natural product (NP) biosynthesis, and to test the practicality of inducing said biosynthesis with different bEBPs. As previously stated, bEBPs are proteins that activate RNAP transcription by binding to  $\sigma^{54}$  unit, which subsequently leads to the expression of  $\sigma^{54}$  dependant BGCs.<sup>30,31</sup>

The model strain for this work, *Photorhabdus laumondii* DSM 15139, contains a large number of BGCs for a *Gammaproteobacteria* (Figure 3). To confirm whether *it* was suitable for testing, we conducted a BLASTp analysis using the core (C) domains DctD (DctD<sub>(141-394)</sub>), a well-studied  $\sigma^{54}$ -dependent bEBP. This identified six similar bEBPs (Table 7), four of which contain the  $\sigma^{54}$ -interacting sequence GAFTGA<sup>31</sup>. The other two, TyrR and PrpR, were included in this work to serve as controls for  $\sigma^{54}$  activation and determine their effect on NP expression.

We have modified five of six identified bEBPs to be constitutively active, generally by removing their regulatory (R) domain (Table A5). For three of these mEBPs, two deletions were obtained: the first involved removing the known R elements, and the second involved removing the full sequence before the C domain. Activation of PspF required only the addition of a start codon, while GlnG was not considered in this work.

We constructed recombinant plasmids containing all pARO190-mEBPs, including DctD<sub>(141-394)</sub>, and conjugated them in *P. laumondii* TTO1 to generate the different variants. However, pARRO190-PrpR<sub>( $\Delta$ 2-208)</sub> was an exception, as multiple attempts at conjugation were unsuccessful. As a result, we were unable to proceed with assessing the effect of PrpR bEBPs on NP biosynthesis in *P. laumondii* TTO1. I suspect that PrpR<sub>( $\Delta$ 2-208)</sub> may have activated the expression of compounds associated with lethal genes<sup>44</sup>, which proved fatal to *P. laumondii*. Basal expression of PrpR<sub>( $\Delta$ 2-208)</sub>, leaked by the *lac* operon<sup>45</sup> controlling it, could have been sufficient to

kill the strain. This could explain why no conjugants were obtained compared to other mEBPs tested. Further experiments using a tightly controlled promoter like *ara* for instance<sup>45,46</sup>, are required to verify this hypothesis.

To evaluate the effect of these mEBPs on NP expression, we selected six compounds: MVAP-A, PL-A, AQ-270, IPS, PPY-D and GXP-A. These compounds were chosen to represent the diverse array of NPs produced by *P. laumondii* and possess documented structures (Table 9 and Figure 14). LC-MS analyses were performed on the variants' metabolite extracts in positive mode. Subsequently, the acquired data were processed using Thermo XCalibur Qual Browser and Compound Discoverer 3.3. Expression levels of the selected compounds were then examined and normalized relative to those of *P. laumondii*-pARO190 for comparison.

DctD<sub>(141-394)</sub>,  $\sigma^{54}$ -dependent pan-activating bEBP generated by Xu *et al.*<sup>35</sup> was used to assess whether  $\sigma^{54}$ -dependent bEBPs could regulate natural product (NP) biosynthesis in *P. laumondii*. From their *in vitro* study, Xu *et al.* concluded that the C domain of DctD interfered with transcription elongation. They reported that those transcripts were terminated shortly after activation<sup>35</sup>. However, my *in vivo* results indicate an increase in metabolite expression in its presence (Figure 5). In the chromatogram of the DctD<sub>(141-394)</sub> variant, metabolite expression exceeds the levels of both *P. laumondii* TTO1 and *P. laumondii*-pARO190. As depicted in Figure 12, DctD<sub>(141-394)</sub> behaved as expected, activating the expression in a statistically significant manner in five out of the six compounds tested. The highest value was observed for PL-A, a 320%-fold change relative to the *P. laumondii*-pARO190.

Amongst the results displayed in Figure 5 and 12, we observed that the *P. laumondii*-pARO190 had an unexpected effect, activating the expression of NPs beyond the levels of the *P. laumondii* TTO1 strain. Sequencing of the empty pARO190 plasmid revealed no unexpected genetic material

(Figure 2). It was reported that during conjugation, recipient cells activate transiently their SOS response system, triggered by the increase in plasmid single-stranded DNA, resulting in an elevated mutation rate and a decrease in fitness<sup>47,48</sup>. pARO190 is a high copy plasmid replicating at a rate of 500-700 copy per cell<sup>49</sup>. Its presence at such a high concentration could have caused the activation in expression witnessed. However, further investigations are needed to determine the exact cause and its effect on growth rate. Taking this into account, we proceeded with the subsequent experiments.

The effect of mEBPs on metabolites and NPs expression were diverse and sometimes unexpected. Some variants exhibited different phenotypes despite having the same mEBP (GlrR( $\Delta$ 2-131), TyrR( $\Delta$ 2-123) and PLU\_RS06090 ( $\Delta$ 2-320), Table 8 and Figures 13 and 14). This discrepancy could be due to either phenotypic heterogeneity, which is well documented in *Photorhabdus*, suggesting that these isogenic bacteria modulated their gene expression in a homogeneous environment<sup>7,26,50</sup>. Alternatively, this could mean that a mutation had occurred in the bacterial genome or the recombinant plasmid. Further enquiry to determine the cause of these phenotypic differences is required. Given the need for additional examination, only the general activation trend of the GlrR( $\Delta$ 2-131), and PLU\_RS06090 ( $\Delta$ 2-320) variants will be retained for further evaluation (Table 10).

The effects of the different mEBPs were compared to that of DctD<sub>(141-394)</sub>, which served as standard for activated expression in *P. laumondii*. Also, as previously stated all results were standardised to the expression levels of *P. laumondii*-pARO190. Our findings reveal new and distinct results for each constitutively active mEBP: TyrR severely repressed expression, whereas PspF and GlrR generally activated expression above the levels of DctD<sub>(141-394)</sub>. Additionally, PspF exhibited some propensity in expressing specific NPs. For PLU\_RS06090 we observed what appeared to be two functions involving both activation and repression, dependent on the extent of

the deleted regulatory region. Next, we will proceed with detailed analysis of the results obtained for each of these mEBPs.

**Table 10.** Summary of findings for each constrictively active EBP.

mEBPs	Size	color	Metabolite expression	NPs expression	Effect on expression	Observed function
<b>DctD</b> <sub>(141-394)</sub>	normal	beige	+	+	Activation	Activator
<b>PspF-M</b>	normal	beige	+	+	Activation	Activator
<b>GlrR</b> <sub>(<math>\Delta</math>2-122)</sub>	normal	beige	++	+	Activation	Activator
<b>GlrR</b> <sub>(<math>\Delta</math>2-131)Y</sub>	normal	yellow	++	+++	Activation	
<b>GlrR</b> <sub>(<math>\Delta</math>2-131)B</sub>	normal	beige	++	+	Activation	
<b>PLU_RS06090</b> <sub>(<math>\Delta</math>2-304)</sub>	normal	beige	-	+/-	Repression	
<b>PLU_RS06090</b> <sub>(<math>\Delta</math>2-320)Y</sub>	normal	yellow	++	+	Activation	Two functions (activator and repressor)
<b>PLU_RS06090</b> <sub>(<math>\Delta</math>2-320)B</sub>	normal	beige	++	+	Activation	
<b>TyrR</b> <sub>(<math>\Delta</math>2-200)</sub>	small	yellow	--	--	Stringent repression	Stringent repressor
<b>TyrR</b> <sub>(<math>\Delta</math>2-123)Y</sub>	normal	yellow	+	+	Activation	
<b>TyrR</b> <sub>(<math>\Delta</math>2-123)B</sub>	normal	beige	-	-	Repression	

All phenotypes are compared to DctD<sub>(141-394)</sub> which is considered an activator (+). All (-) score reflects values below that of *P. laumondii*-pARO190 based on observations from Table 8. For Metabolite expression, this qualitative score was based on Figure 13. For NPs expression, this quantitative score was based on Figure 14. N/A implies that the data cannot be used, further experiments are required.

The transcriptional regulatory protein TyrR is well studied in *Escherichia coli*, where it is known to regulate both repression and activation of gene expression<sup>51,52</sup>. It is reported to play an important role in virulence, biofilm formation<sup>52</sup>, and the biosynthesis and transport of aromatic amino acids<sup>52,53</sup>. In *E. coli* TyrR is recognised as being an unusual bEBP due to the absence of the GAFTGA sequence motif in its C domain, and it was shown to regulate expression through  $\sigma$ 70-dependent RNAP holoenzyme instead<sup>31</sup>. While its role in gene expression regulation in *P. laumondii* has not been extensively explored, it has been shown to play a role in the regulation of stilbene based antibiotics expression<sup>54,55</sup>.

Our results have revealed a previously unreported effect of TyrR<sub>( $\Delta$ 2-200)</sub> mEBP on transcription regulation in *P. laumondii*. This variant displayed the most impactful phenotypic

change, with the formation of small, yellow, slow growing colonies, with near-complete repression of metabolites including NPs expression (Figures 13 and 14). These findings suggest that TyrR may play an important role during the M-Form of *Photorhabdus* lifecycle, as similar phenotypes are observed during that stage<sup>27</sup>.

The bEBP GlrR is reported to negatively regulate genes associated to inorganic polyphosphate synthesis in *E. coli*<sup>56</sup>. Additionally, it plays a role in transcriptional interference by repressing expression from  $\sigma^{70}$ -dependent promoters in both *Salmonella* Typhimurium and *E. coli*<sup>57</sup>. In the case of *P. laumondii*, Gopel *et al.* suggested that increased GlrR activity leads to the enhanced expression of sRNA, which regulates the expression of the GlmS enzyme responsible for producing an essential component of the bacterial cell wall<sup>58</sup>. Our results indicated that constitutively activating GlrR has a significant activating effect on metabolite and NP expression in *P. laumondii*, which was previously undocumented (Figures 13 and 14). This demonstrates great potential in activating the expression of previously undetected NPs.

PspF, or Phage shock protein F, is a well-known transcription factor that operates as part of a response system to inner membrane (IM) stress<sup>59,60</sup>. In *E. coli*, this bEBP is constitutively active (it lacks an R domain) but negatively regulated by PspA<sup>31,60,61</sup>. Upon IM damage, this regulation is lifted, allowing PspF to activate the expression of *psp* genes, initiating repairs and thus preserving energy production by maintaining the balance in the proton motive force<sup>17,58</sup>. However, our bioinformatic analysis indicates that in *P. laumondii* TTO1, PspF remains inactive due to a point mutation in the start codon. This suggests that a point mutation would be necessary for this stress response system to function, limiting its effects to the subset of the population that would naturally carry the mutation.<sup>7,62</sup>

According to our findings, constitutively active PspF activates the expression of metabolites in general, although not to the extent observed with GlrR. However, it demonstrates accentuated expression in *P. laumondii* of certain analyzed NPs: anthraquinone 270, gameXpeptide A and mevalagmapeptide A (Figure 14). This suggests that PspF could be utilized to target the biosynthesis of specific NPs.

PLU\_RS06090 is identified as  $\sigma^{54}$ -interacting transcriptional regulator found in *P. laumondii*. Little is known about the regulatory functions of this bEBP. Our results indicate that depending on the deleted region of the R domain, PLU\_RS06090 exhibits different regulatory functions. When only the R elements are deleted ( $\Delta$ 2-304) (Table A5), it represses the expression of most metabolites and NPs (Figures 13 and 14). However, when the entire region before the C domain ( $\Delta$ 2-320) is removed, we observe a general activation in metabolite expression (Figure 13). These new findings suggest that PLU\_RS06090 bEBP may have two functions, positively and negatively regulating genes involved in the synthesis of a wide range of metabolites including NPs. Moreover, it seems that the stretch of 16 amino acids that lies between the two deletions, codons 304 to 320, plays an important role in mediating these functions.

Our results (Table 10) highlight the efficacy of mEBPs as a tool for NP expression in *P. laumondii*. In contrast to methods like CRAGE and CRAGE-CRISPR, which successfully target specific BGCs using heterologous expression or sequential homologous recombination with specific single-guided RNA<sup>1,24</sup>, our approach stands out for its ability to simultaneously target a broad range of BGCs. By harnessing the innate capacity of the native strain for secondary metabolite biosynthesis and regulation, we avoid the need for complex processes like homologous recombination, ensuring ease of implementation and scalability.

This study has uncovered many new and valuable findings that would serve as milestones in enhancing our understanding of bEBPs' regulatory function in *P. laumondii*. However, we acknowledge that certain experiments are needed to make it comprehensive. This include reducing the levels of the mEBPs expressed in the cell to ensure that the expression observed was mediated by their binding to the UAS elements present upstream of their respective genes. Also, testing the remaining 2 bEBPs, GlnG and PrpR; investigating pARO190 effect and the phenotypic variations observed within the same mEBPs; as well as performing a *rpoN* knockout experiment (for which the knockout cassettes have already been constructed) to confirm that GlrR, PspF and PLU\_RS06090 alter metabolite expression via interactions with  $\sigma^{54}$ .

Some of these experiments are currently in progress. Marcus Simoes (MSc. candidate) is currently assessing the GlnG bEBP and conducting the *rpoN* knockout experiment using the same procedure employed in this study. Investigations regarding the PrpR( $\Delta 2-208$ ) mEBP, pARR190 effect, and the observed phenotype discrepancies within the same mEBP (GlrR( $\Delta 2-131$ ), TyrR( $\Delta 2-123$ ) and PLU\_RS06090 ( $\Delta 2-320$ )), will be assigned to future MSc. students.

The broader goal of this approach was to uncover for new NPs by activating the expression of cryptic BGCs using constitutively active bEBPs. Undergraduate students have already performed extensive screening through spot-on lawn assays to identify new bioactive compound(s), and viability assays are currently underway to assess compound toxicity. The next phase will involve conducting real-time PCR to identify if any cryptic BGCs were expressed.



In summary, our results confirm that  $\sigma^{54}$ -dependent bEBPs can effectively induce natural product biosynthesis, highlighting the importance of  $\sigma^{54}$  role as a global regulator of numerous metabolites including NPs. Additionally, GlrR and PspF, due to their activating function, hold potential for exploring new NPs with antimicrobial properties, which would aid with the ongoing struggle against antimicrobial resistance. As new findings on *P. laumondii* expression regulation, TyrR was found to acts as a stringent repressor of non-essential metabolite expression, while PLU\_RS06090 was identified to potentially have two functions of repression and activation over a wide range of metabolites.

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## **APPENDIX**

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## Supporting Figures and Tables

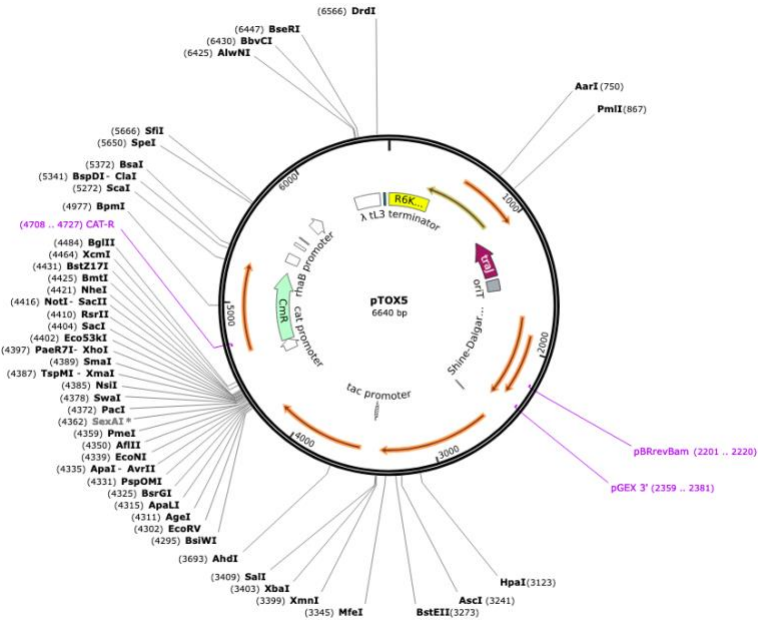


Figure A1. pTOX5 map from Addgene.

Figure A2. Sequence alignment.

Identification of the different bEBPs on *P. laumondii* TTO1 using DctD<sub>(141-394)</sub>. (data obtained using NCBI BLASTp)

GlR bEBP.

MULTISPECIES: two-component system response regulator GlrR [Photorhabdus]						
Sequence ID: <a href="#">WP_011147503.1</a> Length: 445 Number of Matches: 1						
Range 1: 137 to 364 <a href="#">GenPept</a> <a href="#">Graphics</a> <span style="float: right;">▼ Next Match ▲ Previous Match</span>						
Score	Expect	Method	Identities	Positives	Gaps	
225 bits(573) 3e-72 Compositional matrix adjust. 113/228(50%) 152/228(66%) 0/228(0%)						
Query	5	LIGQTPVMERLRQTLKHIADTDVDVLVAGETGSGKEVVATLLHQWSRRRTGNFVALNCGA	64			
		++ ++P M RL + + +A +DV VL+ G++G+GKEV+A ++ S R F+A+NCGA				
Sbjct	137	IVTRSPAMRLRLEQARMVAQSDVSVLINGQSGTGKEVLAQAIHRASPRAKKPFIAINCGA	196			
Query	65	LPETVIESELFHGHEPGAFTGAVKKRIGRIEHASGGTLFLDEIEAMPATQVKMLRVLEAR	124			
		LPE ++ESELFGH GAFTGAV R G A GGTLFLDEI MP A QVK+LRVL+ R				
Sbjct	197	LPEQLLESELFGHAKGAFTGAVSSREGLFLAAQGGTLFLDEIGDMPQALQVKLLRVLQER	256			
Query	125	EITPLGTLNLRPVDIRVVAALKVDLGDPAARGDFREDLYYRLNVVTLIPPLRERRDDIP	184			
		+I PLG+N +D+R+++A DL A+ +FREDLYYRLNVV L IP L R +DIP				
Sbjct	257	KIRPLGNSNRDLIDVRIISATHRDLPKAMAKNEFREDLYYRLNVVNLKIPALNGRVEDIP	316			
Query	185	LLFSHFLARASERFGREVPASAMRAYLATHSWPGNVRELSHFAERV	232			
		LL +H L +++R V + S L SWPGNVR+L + E+				
Sbjct	317	LLANHLRESAKRHKPFVRSFSVDAMKCLMAASWPGNVRQLVNVIEQC	364			

GlnN bEBP.

**MULTISPECIES: nitrogen regulation protein NR(I) [Photorhabdus]**  
 Sequence ID: [WP\\_011144634.1](#) Length: 490 Number of Matches: 1

Range 1: 141 to 365 [GenPept](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Method	Identities	Positives	Gaps
195 bits(496)	3e-60	Compositional matrix adjust.	99/225(44%)	141/225(62%)	0/225(0%)
Query 5	LIGQTPVMERLRQTLKHIADTDVDVLVAGETGSGKEVVATLLHQWSRRRTGNFVALNCGA	64			
	+IG+ P M+ + + + ++ + + VL+ GE+G+GKE+VA LH+ S R F+ALN A				
Sbjct 141	MIGEAPAMQDVYRIIGRLSRSSISVLINGESGTGKELVAHALHRHSPRANEPFIALNMAA	200			
Query 65	LPETVIESELFHGHEPGAFTGAVKKRIGRIEHASGGTLFLDEIEAMPPATQVKMLRVLEAR	124			
	+P+ +IESELFGE GAFTGA + R GR E A+ G+LFLDEI MP Q ++LRVL				
Sbjct 201	IPKDLIESELFGHEKGAFTGANQVRHGRFEQANRGSFLFLDEIGDMPLDIQTRLLRVLAEG	260			
Query 125	EITPLGTNLTRPVDIRVVAAAKVDLGDPAARGDFREDLYYRLNVVTLSSIPLRERRDDIP	184			
	+ +G VD+R++AA DL +G FREDLY+RLNV+ + +PPLR+R +DIP				
Sbjct 261	QFYRVGGYAPVKVDVRIIAATHQDLERLVQKGFREDLYHRLNVIRMQLPPLRDRVEDIP	320			
Query 185	LLFSHFLARASERFGREVPASAMRAYLATHSWPGNVRELSHFA 229				
	L HFL + ++ G E + L WPGNVR+L +				
Sbjct 321	RLTRHFLQQTAKELGVETKILHPDAEIALMRLPWPGNVRQLENIC 365				

TyrR bEBP.

**MULTISPECIES: transcriptional regulator TyrR [Photorhabdus]**  
 Sequence ID: [WP\\_0111446802.1](#) Length: 527 Number of Matches: 1

Range 1: 206 to 439 [GenPept](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Method	Identities	Positives	Gaps
186 bits(471)	2e-56	Compositional matrix adjust.	92/234(39%)	143/234(61%)	0/234(0%)
Query 5	LIGQTPVMERLRQTLKHIADTDVDVLVAGETGSGKEVVATLLHQWSRRRTGNFVALNCGA	64			
	+I +P M ++ + + +A D +L+ GETG+GK+++A H S R F+ LNC +				
Sbjct 206	IIAVSPKMAQIEQARKMAMLDAPLLIGETGTGKDILAKACHLSLRGKQPFLGLNCAS	265			
Query 65	LPETVIESELFHGHEPGAFTGAVKKRIGRIEHASGGTLFLDEIEAMPPATQVKMLRVLEAR	124			
	+P+ V+ESELFG+ GA+ A++ + G E A+GGT+ LDEI M P Q+K+LR L				
Sbjct 266	MPDDVVESELFGYAAGAYPNAIEGKGFQANGGTVLLDEIGEMSPQMQIKLLRFLNDG	325			
Query 125	EITPLGTNLTRPVDIRVVAAAKVDLGDPAARGDFREDLYYRLNVVTLSSIPLRERRDDIP	184			
	+G V++RV+ A + +L + RG+FREDLYYRLNV+ +++P LR+R+ DI				
Sbjct 326	TFRRVGEENEVKVNRVICATQKNLVELVQRGEFREDLYYRLNVLAITLPSLRDRKTDIM	385			
Query 185	LLFSHFLARASERFGREVPASAMRAYLATHSWPGNVRELSHFAERVALGVEG 238				
	L +F+ R SE G P +S + +L ++SWPGNVR+L + R +E				
Sbjct 386	PLVEYFIERFSEEQGVKPKLSPELSHFLTSYSWPGNVRQLQNAIYRALQLEN 439				

PspF bEBP.

**MULTISPECIES: phage shock protein operon transcriptional activator [Photorhabdus]**  
 Sequence ID: [WP\\_011146808.1](#) Length: 334 Number of Matches: 1

Range 1: 8 to 235 [GenPept](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Method	Identities	Positives	Gaps
179 bits(455)	7e-56	Compositional matrix adjust.	96/228(42%)	134/228(58%)	1/228(0%)
Query 5	LIGQTPVMERLRQTLKHIADTDVDVLVAGETGSGKEVVATLLHQWSRRRTGNFVALNCGA				64
Sbjct 8	L+G+ + + + +A V+V GE G+GKE++A LH S R G F++LNC A LLGEANSFLEILEQVSALAKLSKPVIVIGERGTGKELIARRLHYLSPRWQGPFI LNCAA				67
Query 65	LPETVIESEELFGHEPGAFTGAVKKRIGRIEHASGGTLFLDEIEAMPPATQVKMLRVLEAR				124
Sbjct 68	L E +++SEELFGHE GAFTGA K+ GR E A GGTLFLDE+ P Q K+LRV+E LNENLLDSEELFGHEAGFTGAQKRHQGRFERADGGTLFLDELATAPMQVQEKLLRVIEYG				127
Query 125	EITPLGTLNLRPVDIRVVAAAKVDLGDPAARGDFREDLYRNLNVVTL SIPPLRERRDDIP				184
Sbjct 128	+ +G + + VD R++ A +L AA+G FR DL RL ++IPPLR+R+ DI HLERVGGSQLQVDARLICATNDNLPAMAAGQFRADLLDRLAFDVVNIIPPLRQQDIM				187
Query 185	LLFSHFLARASERFGREV-PAISAAMRAYLATHSWPGNVRELSHFAER				231
Sbjct 188	LL HF + + P + R L ++WPGN+REL + ER LLAQHFAIQMCRELDLPLFPGFTDQARQLQNYTWPGNIRELKNVVER				235

PrpR bEBP.

**MULTISPECIES: propionate catabolism operon regulatory protein PrpR [Photorhabdus]**  
 Sequence ID: [WP\\_011147723.1](#) Length: 555 Number of Matches: 1

Range 1: 226 to 466 [GenPept](#) [Graphics](#) [Next Match](#) [Previous Match](#)

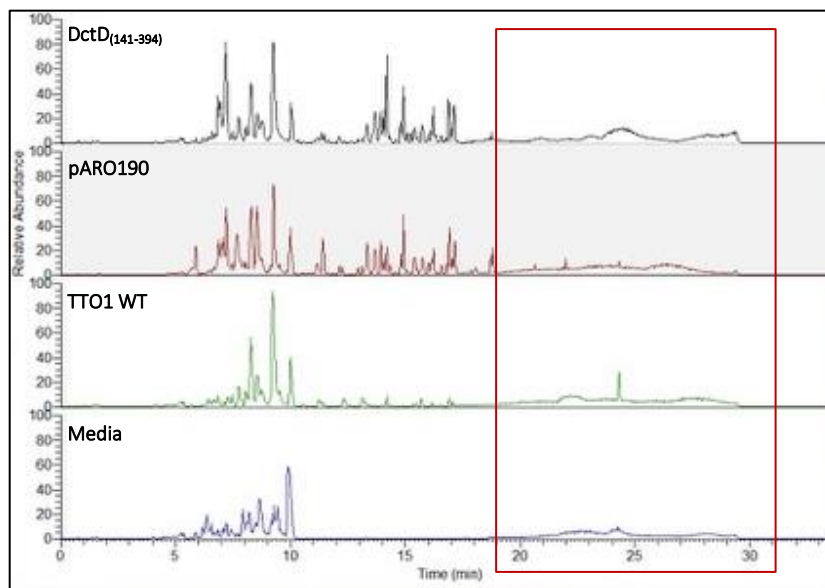
Score	Expect	Method	Identities	Positives	Gaps
176 bits(445)	2e-52	Compositional matrix adjust.	103/245(42%)	144/245(58%)	21/245(8%)
Query 7	GQTPVMERLRQTLKHIADTDVDVLVAGETGSGKEVVATLLHQWSRRRTGN-----FV				58
Sbjct 226	G + VME +R T+ A + VL+ GE+G+GKE+VA +H R G FV GDSAVMEHVRNTIMLYARSPATVLIQGESGTGKELVAQAIHHEYALRYGQVAGKHQRPV				285
Query 59	ALNCGALPETVIESEELFGHEPGAFTGAVKK-RIGRIEHASGGTLFLDEIEAMPPATQVKM				117
Sbjct 286	A+NCGA+ E+++E+ELFG+E GAFTG+ + R G E A GTLFLDEI MP Q ++ AVNCGAIAESLLEAELFGYEEGAFTGSRGGRAGLFEVAHRGTLFLDEIGEMPLPLQTRL				345
Query 118	LRVLEAREITPLGTLNLRPVDIRVVAAAKVDLGDPAARGDFREDLYRNLNVVTL SIPPLR				177
Sbjct 346	LRVLE + + +G + VD+R++ A DL G FR DL+YRL+V+ ++IP LR LRVLEEKSVVRVGGHRPIAVDVRIICATHCDLDTWVQDGRFRTDLFYRLSVLRINIPALR				405
Query 178	ERRDDIPLLFSHFLARA-----SERFGREVPAISAAMRAYLATHSWPGNVRELSHFA				229
Sbjct 406	ER DI +L +L RA S+ +E+ A++AY WPGNVREL + ERGHDIGILALEYLRRFAALNLPVSRVQELATCQEALQAY----HWPGNVRELRLNM				461
Query 230	ERVAL 234				
Sbjct 462	ER+ L ERLTL 466				

PLU\_RS06090 bEBP.

**MULTISPECIES: sigma 54-interacting transcriptional regulator [Photorhabdus]**  
 Sequence ID: [WP\\_011145558.1](#) Length: 643 Number of Matches: 1

Range 1: 324 to 550 [GenPept](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Method	Identities	Positives	Gaps
194 bits(494)	8e-59	Compositional matrix adjust.	97/229(42%)	144/229(62%)	4/229(1%)
Query 5	LIGQTPVMERLRQTLKHIADTDVDVLVAGETGSGKEVVATLLHQWSRRRTGNFVALNCGA	64			
	+IG++ + ++ + +K +A TD +V++ GE+G+GKE++A +HQ S R+ + +NC A				
Sbjct 324	IIGRSASILQIEKIKVAPTANVMIYGESGTGKELIARAIHQSSHRKQQLIRVNCAA	383			
Query 65	LPETVIESELFGHEPGAFTGAVKKRIGRIEHASGGTLFLDEIEAMPPATQVKMLRVLEAR	124			
	+P + ESE FGH GAFTGAV+ R GR E A GTLFLDEI +P Q K+LRVL+				
Sbjct 384	IPSELFEESEFFGHVKGAF TGAVRDRAGRFELADQGTFLFLDEIGEIPIELQSKLLRVLQEG	443			
Query 125	EITPLGTNLTRPVDIRVVAAAKVDLGDPAARGDFREDLYYRLNVVTL SIPLRERRDDIP	184			
	+G TR VD+R+++AA +L + FREDLY+RLNV + P LR+R++DIP				
Sbjct 444	TFERVGEEKTRHVDVRIIAATNRNLKEEVKHKRFREDLYFRLNVFPIYSPALRDRKEDIP	503			
Query 185	LLFSHFLARASERFGREVP AISAMRAY--LATHSWPGNVRELSHFAER	231			
	LL +HF ++ R++ + + R L + WPGN+REL + ER				
Sbjct 504	LLVTHFTKLICDK--RKINYL PFSQRHILELQQYDWPGNIRELQNVIER	550			



**Figure A3. Background noise.**

Chromatograms of different samples indicating the presence of background noise. The y axis indicates the relative abundance (PH) at a scale of **7.00 E8**. The x axis indicates the elution time in minutes of the different metabolites. The red box indicates the area from 19 min on, that displays strong background noise and indistinguishable peaks. Data from Xcalibure analysis software.

**Table A1.** Primers used to confirm bEBPs' presence in *P. laumondii* TTO1.

Primers	Sequences
FW- <i>pspF</i>	TGGGTGAAGCAAACAGCTTTC
RC- <i>pspF</i>	AGTCGTAGTTTCTCTGCTGCTT
FW- <i>PLU RS06090</i>	GAACACACGTCACAGGCATT
RC- <i>PLU RS06090</i>	ATTTTGCTGCGCCATCATCA
FW- <i>glrR</i>	CGTGTACAACCCGACAGAAAG
RC- <i>glrR</i>	GAATTCAGTCCGGTTACGCC
FW- <i>tyrR</i>	GCGCTTAGAAGTTGTTTGCCA
RC- <i>tyrR</i>	TTTAGCCAGTTTACGCGTGC
FW- <i>prpR</i>	GGACAGTCTCTGTTTCCCGT
RC- <i>prpR</i>	GATATTCCGCGGTTGCATGG

All primers were ordered from IDT DNA. Abbreviations, FW: forward primer and RC: reverse complement primer

**Table A2.** Knockout cassette design.

Name	Function	Modifications & Sequences
<b>rpoN-KC</b>	1 <sup>st</sup> RE EcoRV; 1 <sup>st</sup> HA; J23100 promoter in red; Km <sup>r</sup> ; 2 <sup>nd</sup> HA; 2 <sup>nd</sup> RE PacI	<u>GATATCGCCAGGAGAATTGATTTAACGCTATGAAGCAAAGTTTGCAACTCAGGCTT</u> AGTCAGCAACTGGCTATGACACCACAGCTCCAACAAGCTATTCGTTTGTTtaga <b>GTTG</b> <b>ACGGCTAGCTCAGTCTTAGGTACAGTGCTAGCT</b> tctaga <b>GAAAGAGGGGACA</b> Aactagatg agccatattcaacgggaacgctctgctctaggcccgattaaattccaacatggatgctgatttatatgggtataaatgggctcgcgataat gtcgggcaatcaggtgcgacaatctatcgattgatgggaagcccgatcgcagagattgttctgaaacatggcaaaagtagcgttgcca atgatgttacagatgagatggtcagactaaactgctgacggaattatgcctctccgaccatcaagcattttatccgactcctgatgatgc atggttactaccactgcgatccccgggaaacagcattccaggtattagaagaatctgattcaggtgagaatattgtgatgcgctgg cagttctcgcgctggttcattcgaatcctgtttgtaattgctctttaaacagcgcgctgattctgctcagcgcgcaatcacgaatg aataacggttggtgatgagtgattgatgacgagcgtaatgctgctgctggtgaaacagctgaaagaaatgcataaacttttgcca ttcaccggattcagctgactcatggtgatttctcacttgataacctatgtttgacgaggggaaataggtgtattgatgttgacga gtcgaatcgcagaccgataaccaggtatgccaatcctggaactgctcctggtgagtttctctcattacagaacaggcttttcaaaaat atggtattgataactctgatataaattcagtttcaattgatgctcgcgatgatttttctaaagTTGCCCGACGAACGTGTCGC AAAATATCGAGAGTCGTTATCCATTCCGCCATCAAACCAACGTAGGCGTCTGGTTT GAAATGACAGAGAAGGAACAAATTTAATTAACGC
<b>Plu1113-KC</b>	1 <sup>st</sup> RE EcoRV; 1 <sup>st</sup> HA; J23100 promoter in red; Km <sup>r</sup> ; 2 <sup>nd</sup> HA; 2 <sup>nd</sup> RE PacI	GGGGATATCAATTAATGATATTATCCTCAGCTCAATGAAATAGAAAGTAATAATAA ATAGTTATGATAACTATAGGATTAAACACTTAAAAAACAATAAAATTTATTtaga <b>G</b> <b>TTGACGGCTAGCTCAGTCTTAGGTACAGTGCTAGCT</b> tctaga <b>GAAAGAGGGGACA</b> Aact agatgagccatattcaacgggaacgcttctgctcagcccgattaaattccaacatggatgctgatttatatgggtataaatgggctcgcg ataatgctcggcaatcaggtgcgacaatctatcgattgatgggaagcccgatcgcagagattgttctgaaacatggcaaaagtagcgt tgccaatgatgttacagatgagatggtcagactaaactgctgacggaattatgcctctccgaccatcaagcattttatccgtactcctgat gatgcatggttactcaccactgcgatccccgggaaacagcattccaggtattagaagaatctgattcaggtgagaatattgtgatgc gctggcaggttctcgcgctggttcattcgaatcctgtttgtaattgctctttaaacagcgcgctgattctcctcagcgcgcaatcac gaaatgaataacggttggtgatgagtgattttgatgacgagcgtaatggctgctgctgaaacagctgaaagaaatgcataaacttt tgccaattcaccggattcagctgactcatggtgatttctcacttgataacctatgtttgacgaggggaaataggtgtattgatgttg acgagtcggaaatcgcagaccgataaccagatctgccaatcctatggaactgctcctggtgagtttctcctcattacagaacggcttttca aaaatattggtattgataatcctgatataaattgcagtttcaattgatgctcgcgatgatttttctaaagGCCTGTCTTTCCTACA CAAATATTTTAAACCCGCCCGCTTATTGAACTTTTCCCTCATAGATTGATCTCAA TAAAAAACACGACTTGAGGTAATCTTTTAAATTAACGC
<b>Plu1213-KC</b>	1 <sup>st</sup> RE EcoRV; 1 <sup>st</sup> HA; J23100 promoter in red; Km <sup>r</sup> ; 2 <sup>nd</sup> HA; 2 <sup>nd</sup> RE PacI	<u>GATATCGTGGATAAAAAAATGACAACATATGAAGCTGATTGATCGTGAATACTGGCT</u> GATGCGGGTTGGTGAATTTAACTAAGGCCGCTGCCACAACCCGACAGATTTtaga <b>GTTG</b> <b>ACGGCTAGCTCAGTCTTAGGTACAGTGCTAGCT</b> tctaga <b>GAAAGAGGGGACA</b> Aactagat agccatattcaacgggaacgctctgctcagcccgattaaattccaacatggatgctgatttatatgggtataaatgggctcgcgataa gtcgggcaatcaggtgcgacaatctatcgattgatgggaagcccgatcgcagagattgttctgaaacatggcaaaagtagcgttgcc aatgatgttacagatgagatggtcagactaaactgctgacggaattatgcctctccgaccatcaagcattttatccgactcctgatgatg catggttactcaccactgcgatccccgggaaacagcattccaggtattagaagaatctgattcaggtgagaatattgtgatgcgctg gcagttctcgcgctggttcattcgaatcctgtttgtaattgctctttaaacagcgcgctgattctcctcagcgcgcaatcacgaatg gaataacggttggtgatgagtgattttgatgacgagcgtaatggctgctgctgaaacagctgaaagaaatgcataaacttttcc attctcaccggattcagctgactcatggtgatttctcacttgataacctatgtttgacgaggggaaataggtgtattgatgttgacga gtcggaaatcgcagaccgataaccaggtatgccaatcctatggaactgctcctggtgagtttctcctcattacagaacaggcttttcaaaaat atggtattgataactctgatataaattcagtttcaattgatgctcgcgatgatttttctaaagGATGGAAATTCGTGCTCA

		ATTTGCAGGTGATGTCATGCCAAAAGAGCAAATAAAAAACGGTCTATCTGAATTTA ATGAACGGATAACTCATAAAAAATTAATTAATTAACGC
<b>Plu2733- KC</b>	1 <sup>st</sup> RE EcoRV; 1 <sup>st</sup> HA; J23100 promoter in red; Km <sup>r</sup> ; 2 <sup>nd</sup> HA; 2 <sup>nd</sup> RE PacI	<u>GATATCCGTGGAGCCGCACAATAATGGAATATAATAACAACTATTGGCCGTTGAAT TATACACAGCTCCGTTAGCTATTGCTAATACTGTTGCTGACGATAAGTC</u> <u>tagaGTTG ACGGCTAGCTCAGTCTAGGTACAGTGTAGCT</u> <u>tctagaGAAAGAGGGGACAA</u> <u>aactagat gagccatattcaacggaaacgctctgcttagccgataaattccaacatggatgctgatttatatgggtataaatgggctcgcgataa tgcgggcaatcaggtgacacaatctatcattgatgggaagcccgatgcccagagttgtttcgaacatgccaaggtagcgttccc aatgatgttacagatgagatggtcagactaaactggctgacggaatttatgctctccaccatcaagcattttaccgtactcctgatgatg catggtactcaccactcgcgacccccgggaaacagcattccaggtattagaagaatctctgattcaggtgagaatattgttgatcgctg gcagttctctgcccgttgcattcattctgttttaattgctctttaacagcagcgcgatttctctcctcagcggcaatcacgaat gaataacgggttgggtgatcgagtgattttgatgacgagcgtaatggctggcctgttgaacaagctcggaaagaaatcataaactttgcc attctcaccggattcagtcgactcatggtgatttctactgataacctattttgacgaggggaaataataggttattgatgttgacga gtcggaaatcgagacgataaccaggatctgcatcctatggaactgctcggtagttttctcctcattacagaacggcttttcaaaaat atggtattgataatctgatatgaataaattcagtttcatttgatgctcaggtttttctaaagTGGGGCTGTTTTTGATTGA TTTATTGGATTGCATAGGCACTCAGCATAACTTGATGCCTGGTGATACGGAACGAT AACAAATCGTAAAAATGGATGCTGATTTAATTAACGC</u>
<b>Plu3534- KC</b>	1 <sup>st</sup> RE EcoRV; 1 <sup>st</sup> HA; J23100 promoter in red; Km <sup>r</sup> ; 2 <sup>nd</sup> HA; 2 <sup>nd</sup> RE PacI	<u>CGATATCAATCACCACCTCGGTTACCTCATATTCTGCCACTACAGCCCCAATTGACTT GTTACGTTGAATAAACCATTCTTTTTCAAGCCAATCACATAACTGTTCTA</u> <u>tagaGTTGA CGGCTAGCTCAGTCTAGGTACAGTGTAGCT</u> <u>tctagaGAAAGAGGGGACAA</u> <u>aactagtgag ccatattcaacggaaacgctctgcttagccgataaattccaacatggatgctgatttatatgggtataaatgggctcgcgataatgc gggcaatcaggtgacacaatctatcattgatgggaagcccgatgcccagagttgtttcgaacatgccaaggtagcgttccaatg atgttacagatgagatggtcagactaaactggctgacggaatttatgctctcctccaccataagcattttaccgtactcctgatgatgctg gttactcaccactcgcgacccccgggaaacagcattccaggtattagaagaatctctgattcaggtgagaatattgttgatcgctggcag tgtcctgcccgggtgattcattcctgtttgaattgctctttaacagcagcgcgatttctcctcagcggcaatcacgaatgaata acgggttgggtgatgagtgattttgatgacgagcgtaatggctggcctgttgaacaagctcggaaagaaatcataaactttgccattctc accgattcagtcgactcatggtgatttctactgataacctattttgacgaggggaaataataggttattgatgttgagcagatcg gaatcgagaccgataaccaggtcttgcctcctatggaactgctcctggtgagttttctcctcattacagaacggcttttcaaaaatag tattgataatcctgatatgaataaattgcagtttcatttgatgctcaggtttttctaaagGGGAAAGTGAGTGCCCTTAAGT TTGAACCTTAACCTACCATCTTTACTAAACAGCAATACTCCCTGCTCGTAAGCATCT TGGACTAATTCAGCATATCCAATTAATTAACGC</u>
<b>KC</b> : knockout cassette; <b>FW</b> : forward primer; <b>RC</b> : reverse complement primer; <b>RE</b> : restriction enzyme; <b>1<sup>st</sup> HA</b> : homologous arm at the gene start; <b>2<sup>nd</sup> HA</b> : homologous arm at the gene end; <b>Km<sup>r</sup></b> : kanamycin resistance.		

**Table A3.** Primers used for the Knockout project.

Name	Function	Modifications & Sequences
<b>FW: rpoN-KC</b>	Amplify the KC	GATATCGCCAGGAGAATTGATTTAACGC
<b>RC: rpoN-KC</b>	Amplify the KC	TTAATTAATTTGTCTTCTTCTCTGTTC
<b>FW: Plu1113-KC</b>	Amplify the KC	GATATCAATTAATGATATTATCCTCAGC
<b>RC: Plu1113-KC</b>	Amplify the KC	TTAATTAATAAAGATTACCTCAAGTCGTG
<b>FW: Plu1213-KC</b>	Amplify the KC	GATATCGTGGATAAAAAAATGACAACATG
<b>RC: Plu1213-KC</b>	Amplify the KC	TTAATTAATTAATTTTTATGAGTTATCCGTTG
<b>FW: Plu2733-KC</b>	Amplify the KC	GATATCCGTGGAGCCGCACAATAATG
<b>RC: Plu2733-KC</b>	Amplify the KC	TTAATTAATCAGCATCCATTTTACGATTTG
<b>FW: Plu3534-KC</b>	Amplify the KC	GATATCAATCACCACCTCGGTTACCTCATATTC
<b>RC: Plu3534-KC</b>	Amplify the KC	TTAATTAATGGATATGCTGAAATTAGTCCAAG
<b>FW: rpoN</b>	Confirm knockout	TCAGGCTTAGTCAGCAACTGG
<b>RC: rpoN</b>	Confirm knockout	TGTTTCATTTCAAACCAGACGCC
<b>FW: Plu1113</b>	Confirm knockout	ATCCTCAGCTCAATGAAATAGAAAAG
<b>RC: Plu1113</b>	Confirm knockout	AGGGAAAAGTTCAATAACGCGG
<b>FW: Plu1213</b>	Confirm knockout	CGTGAATACTGGCTGATGCG
<b>RC: Plu1213</b>	Confirm knockout	TGCTCTTTTGGCATGACATCAC
<b>FW: Plu2733</b>	Confirm knockout	TGGAGCCGCACAATAATGGA
<b>RC: Plu2733</b>	Confirm knockout	CCGATCACCAGGCATCAAGT
<b>FW: Plu3534</b>	Confirm knockout	ATATTCTGCCACTACAGCCCC
<b>RC: Plu3534</b>	Confirm knockout	AAGATGCTTACGAGCAGGGAG
<b>KC</b> : knockout cassette; <b>FW</b> : forward primer; <b>RC</b> : reverse complement primer.		



**Table A4.** 22 BGCs identified in *P. laumondii* TTO1 using antiSMASH (NC\_005126.1)

<b>Region</b>	<b>Most similar known cluster</b>	<b>Similarity</b>
<b>1</b>		
<b>2</b>	1-carbapen-2-em-3-carboxylic acid	41%
<b>3</b>		
<b>4</b>		
<b>5</b>	mevalgmapeptide A/ mevalgmapeptide B/ mevalgmapeptide C/ mevalgmapeptide D	<b>95%</b>
<b>6</b>	endopyrrole B/ endopyrrole A	50%
<b>7</b>	malonomycin	11%
<b>8</b>		
<b>9</b>	O-antigen	14%
<b>10</b>	luminmycin A/ glidobactin A/ cepafungin	100%
<b>11</b>	minimycin	80%
<b>12</b>	HTTPCA/ prepiscibactin/ piscibactin	97%
<b>13</b>	kolossin	100%
<b>14</b>	frederiksenibactin	23%
<b>15</b>	gameXpeptide A/ gameXpeptide B/ gameXpeptide E/ luminmide B/ luminmide D/ luminmide E/ luminmide F/ luminmide G	13%
<b>16</b>	gameXpeptide A/ gameXpeptide B/ gameXpeptide E/ luminmide B/ luminmide D/ luminmide E/ luminmide F/ luminmide G	<b>94%</b>
<b>17</b>		
<b>18</b>	odilorhabdin NOSO-95A/ odilorhabdin NOSO-95B/ odilorhabdin NOSO-95C	70%
<b>19</b>		
<b>20</b>	anthelvencin A/ anthelvencin B/ anthelvencin C	13%
<b>21</b>	carotenoid	83%
<b>22</b>	putrebactin/ avaroferrin	100%
<b>23</b>	conglobatin	10%

**Table A5.** UniProt analysis of the Regulatory domain of *P. laumondii* bEBPs.

<b>bEBPs</b>	<b>Regulatory elements</b>	<b>Codon position</b>
PspF	N/A	N/A
PLU_RS06090	PAS and PAC	169 to 237 and 241 to 292
GlrR	Response regulatory (RR)	8 to 122
TyrR	ACT and PAS	2 to 72 and 78 to 123
PrpR	unidentified regulatory domain	-

**Table A6.** Observation (conjugated TTO1 colonies and LC-MS metabolite extracts).

<b>Samples' name</b>	<b>Colony color</b>	<b>Colony round shape/growth</b>	<b>Volume extracted</b>	<b>Beads color</b>	<b>Extract color</b>	<b>Pellet color</b>
<b>Media</b> (control #4, p182)	N/A	N/A	700ul	-	-	Light yellow
<b>TTO1 WT</b> (Plum TTO1 phase I, p186)	Beige	Round, regular	700ul	Light beige	-	Brown/deep orange
<b>pAro190</b> (pAro190#1, p135)	Beige	Round, regular	-	-	-	-
<b>pAro190</b> (pAro190#2, p135)	Beige	Round, regular	-	-	-	-
<b>pAro190</b> (pAro190#3, p135)	Beige	Round, regular	-	-	-	-
<b>dctD</b> (tDctD/LR 1#1, p179)	Beige	Round, regular	950ul	-	Deep orange	Brown/deep orange
<b>dctD</b> (tDctD/LR 1#2, p179)	Beige	Round, regular	800ul	-	Deep orange	Brown/deep orange
<b>dctD</b> (tDctD/LR 1#3, p179)	Beige	Round, regular	950ul	-	Deep orange	Brown/deep orange
<b>pspF</b> (pspF-M#1, p98)	Beige	Round, regular	850ul	Brown/deep orange	-	-
<b>pspF</b> (pspF-M#2, p98)	Beige	Round, regular	850ul	Brown/deep orange	-	-
<b>pspF</b> (pspF-M#3, p98)	Beige	Round, regular	850ul	Brown/deep orange	-	-
<b>PAS 304</b> (uPAS-D2-304#1, p98)	Beige	Round, regular	850ul	Light orange	-	-
<b>PAS 304</b> (uPAS-D2-304#2, p98)	Beige	Round, regular	850ul	Light orange	-	-
<b>PAS 304</b> (uPAS-D2-304#3, p98)	Beige	Round, regular	850ul	Light orange	-	-
<b>PAS 320-Y</b> (uPAS-D2-320 3-1, p186)	Yellow	Round, regular	700ul	-	Light orange	Brown/deep orange
<b>PAS 320-Y</b> (uPAS-D2-320 3-2, p186)	Yellow	Round, regular	700ul	-	Light orange	Brown/deep orange
<b>PAS 320-Y</b> (uPAS-D2-320 3-3, p186)	Yellow	Round, regular	700ul	-	Light orange	Brown/deep orange
<b>PAS 320-B</b> (uPAS-D2-320 1-1, p186)	Beige	Round, regular	700ul	Orange	Orange	Brown/deep orange
<b>PAS 320-B</b> (uPAS-D2-320 1-2, p186)	Beige	Round, regular	630ul	Orange	Orange	Brown/deep orange
<b>PAS 320-B</b> (uPAS-D2-320 1-3, p186)	Beige	Round, regular	700ul	Orange	Orange	Brown/deep orange
<b>glrR 122</b> (glrR-D2-122 1#1, p179)	Beige	Round, regular	950ul	-	Light orange	Brown/deep orange

<b>glrR 122</b> (glrR-D2-122 1#2, p179)	Beige	Round, regular	950ul	-	Light orange	Brown/deep orange
<b>glrR 122</b> (glrR-D2-122 1#3, p179)	Beige	Round, regular	800ul	-	Light orange	Brown/deep orange
<b>glrR 131-Y</b> (glrR-D2-131 1-1, p186)	Yellow	Round, regular	700ul	-	Deep orange	Brown/deep orange
<b>glrR 131-Y</b> (glrR-D2-131 1-2, p186)	Yellow	Round, regular	700ul	-	Deep orange	Brown/deep orange
<b>glrR 131-Y</b> (glrR-D2-131 1-3, p186)	Yellow	Round, regular	700ul	-	Deep orange	Brown/deep orange
<b>glrR 131-B</b> (glrR-D2-131 5-1, p186)	Beige	Round, regular	700ul	Orange	Light orange	Brown/deep orange
<b>glrR 131-B</b> (glrR-D2-131 5-2, p186)	Beige	Round, regular	700ul	Orange	Light orange	Brown/deep orange
<b>glrR 131-B</b> (glrR-D2-131 5-3, p186)	Beige	Round, regular	700ul	Orange	Light orange	Brown/deep orange
<b>tyrR 123-Y</b> (tyrR-D2-123 5-1, p182)	Yellow	Round, regular	700ul	-	-	Deep orange
<b>tyrR 123-Y</b> (tyrR-D2-123 5-2, p182)	Yellow	Round, regular	700ul	-	-	Deep orange
<b>tyrR 123-Y</b> (tyrR-D2-123 5-3, p182)	Yellow	Round, regular	700ul	-	-	Deep orange
<b>tyrR 123-B</b> (tyrR-D2-123 6-1, p182)	Beige	Round, regular	700ul	-	-	clear yellow
<b>tyrR 123-B</b> (tyrR-D2-123 6-2, p182)	Beige	Round, regular	700ul	-	-	clear yellow
<b>tyrR 123-B</b> (tyrR-D2-123 6-3, p182)	Beige	Round, regular	700ul	-	-	clear yellow
<b>tyrR 200</b> (tyrR-D2-200#1, p98)	Yellow	Very small/slow	850ul	Yellow	-	-
<b>tyrR 200</b> (tyrR-D2-200#2, p98)	Yellow	Very small/slow	850ul	Yellow	-	-
<b>tyrR 200</b> (tyrR-D2-200#3, p98)	Yellow	Very small/slow	850ul	Yellow	-	-

**Table A7.** The compounds abundance (Raw data of corrected average of PH (e7))

Variants	PL-A	AQ 270	PPY-D	GXP-A	IPS	MVAP-A
<b>DctD</b> <sub>(<math>\Delta</math>141-394)</sub>	6.18	1.24	8.67	11.24	20.03	60.89
<b>P.lau-pARO190</b>	1.93	0.58	5.32	9.50	18.87	38.93
<b>P.lau WT</b>	0.88	0.24	4.21	6.61	1.91	7.19
<b>pspF-M</b>	3.89	1.49	6.78	14.12	17.10	81.40
<b>PLU_RS06090</b> <sub>(<math>\Delta</math>2-304)</sub>	0.18	0.26	0.72	7.86	1.13	1.26
<b>PLU_RS06090</b> <sub>(<math>\Delta</math>2-320)</sub> <b>B</b>	6.05	1.19	4.13	19.82	34.80	64.17
<b>PLU_RS06090</b> <sub>(<math>\Delta</math>2-320)</sub> <b>Y</b>	3.86	0.66	11.55	18.05	27.14	88.44
<b>glrR</b> <sub>(<math>\Delta</math>2-122)</sub>	5.05	0.96	9.72	14.24	20.45	69.06
<b>glrR</b> <sub>(<math>\Delta</math>2-131)</sub> <b>Y</b>	6.85	1.33	12.41	17.42	25.65	89.48
<b>glrR</b> <sub>(<math>\Delta</math>2-131)</sub> <b>B</b>	4.87	0.81	10.01	16.15	31.30	69.85
<b>tyrR</b> <sub>(<math>\Delta</math>2-123)</sub> <b>Y</b>	3.80	0.88	13.13	13.25	21.13	79.53
<b>tyrR</b> <sub>(<math>\Delta</math>2-123)</sub> <b>B</b>	0.14	0.00	2.09	11.68	1.17	0.34
<b>tyrR</b> <sub>(<math>\Delta</math>2-200)</sub>	0.00	0.00	0.00	4.82E-03	0.00	0.00